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Melatonin and Environmental Magnetic Fields

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13. ABSTRACT (Maximum 200 words) <p>The overall purpose of this study is to investigate the role that environmental-level magnetic fields may play as an exogenous factor in the etiology of human breast cancer. During this two-year research period significant progress has been made. We have observed that a) the hormone melatonin and drug tamoxifen, which both inhibit human breast cancer cell growth, are each blocked or inhibited by environmental-level 12 mG (60Hz) magnetic fields; b) this effect is observed at physiological levels of melatonin and pharmacological levels of tamoxifen; c) an exposure dose-response exists between 2-12 mG for melatonin; d) exposure duration data indicates field treatment is required for at least one cell cycle; e) the magnetic field not the electric field is the operative field metric; and f) frequency dependency experiments suggest this interaction is consistent with relatively long times of milliseconds implicating slow biological-based processes. Of significant importance are two recent reports by independent laboratories at the EPA and at the University of California, Riverside, which present independent confirmation of our original melatonin findings.</p>				
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FOREWORD

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Robert P. Flendy June 27 1977
PI - Signature Date

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Introduction.

This report represents the third report submitted for this two year research project. There have been two previous annual reports that have been submitted and accepted, and this last report covers new research conducted during a no-cost extension for the period April - June 1997, as well as including a summarization of some of our first and second year findings. Please refer to the two previous annual reports which present and discuss, in some detail, our research progress for Year 1 and Year 2.

The overall purpose of this study is to investigate the role that environmental-level magnetic fields may play as an exogenous risk factor in the etiology of human breast cancer. We are testing the hypothesis that an environmental-level 12 mGauss 60 Hz magnetic field influences hormone (melatonin) and drug (tamoxifen) interactions with human breast cancer cells. This approach is based on our published findings that a 12 mGauss (60 Hz, sinusoidal) magnetic field (a) blocks the cytostatic action of melatonin and (b) new findings that the same 12 mGauss magnetic field significantly inhibits the cytostatic action of tamoxifen on MCF-7 breast cancer cell growth. As indicated in our proposal we will undertake biophysically-based (Phase I) and biologically-based (Phase II) in vitro studies to investigate these interactions. Our goal is to understand these interactions from the point of field coupling (biophysical insights) and cellular responses (biological insights).

Body.

Progress in addressing Phase I and Phase II Specific Aims, discussed below, are summarized in this section. Further details about our scientific findings can be found in our two previous annual reports for Y1 and Y2.

Phase I: Biophysically-Based Studies.

1. (Y1) *Determine the magnetic field dose threshold. We have observed that a 12 mGauss magnetic field blocks melatonin's oncostatic function, but a 2 mGauss field does not. A dose-threshold should exist between 2 - 12 mGauss and we will attempt to identify this threshold.*

Studies conducted during the recent no-cost extension have established a that a dose-response exists between 2 - 12mG for blocking of melatonin's natural oncostatic action, and that an optimal dose of 12mG exists for tamoxifen. A high priority was set to obtain this information by the reviewers in the last critique (dated January 7, 1997). We thus emphasized this need in our research effort.

In these experiments we employed MCF-7 cells of the same passage which were split into identical cell aliquots and then seeded into plates placed into matched incubators operating at 2, 6, 10, 12, 20, or 1000 mGauss(60Hz). Cells in each incubator were treated with melatonin at 10^{-9} M or not. Refer to our experimental design shown in Figure 1 and described in detail in previous reports. Cell growth was followed over days 5 - 7 corresponding to exponential growth and the percentage change observed for melatonin treatment vs. no melatonin at the above field intensities was determined.

Figure 2 displays this dose-response data. In this figure "n" refers to the number of independent experiments conducted at each field intensity. In these studies we included 2 and 12 mGauss treatment groups when other field intensities were employed so we could determined that a) melatonin inhibited cell growth in a 2 mGauss field which verified that the cells were biologically active and appropriately responding to melatonin, and b) that a 12 mGauss field inhibited this melatonin effect, which corresponds to the magnetic field bioeffect we have

Experimental Design

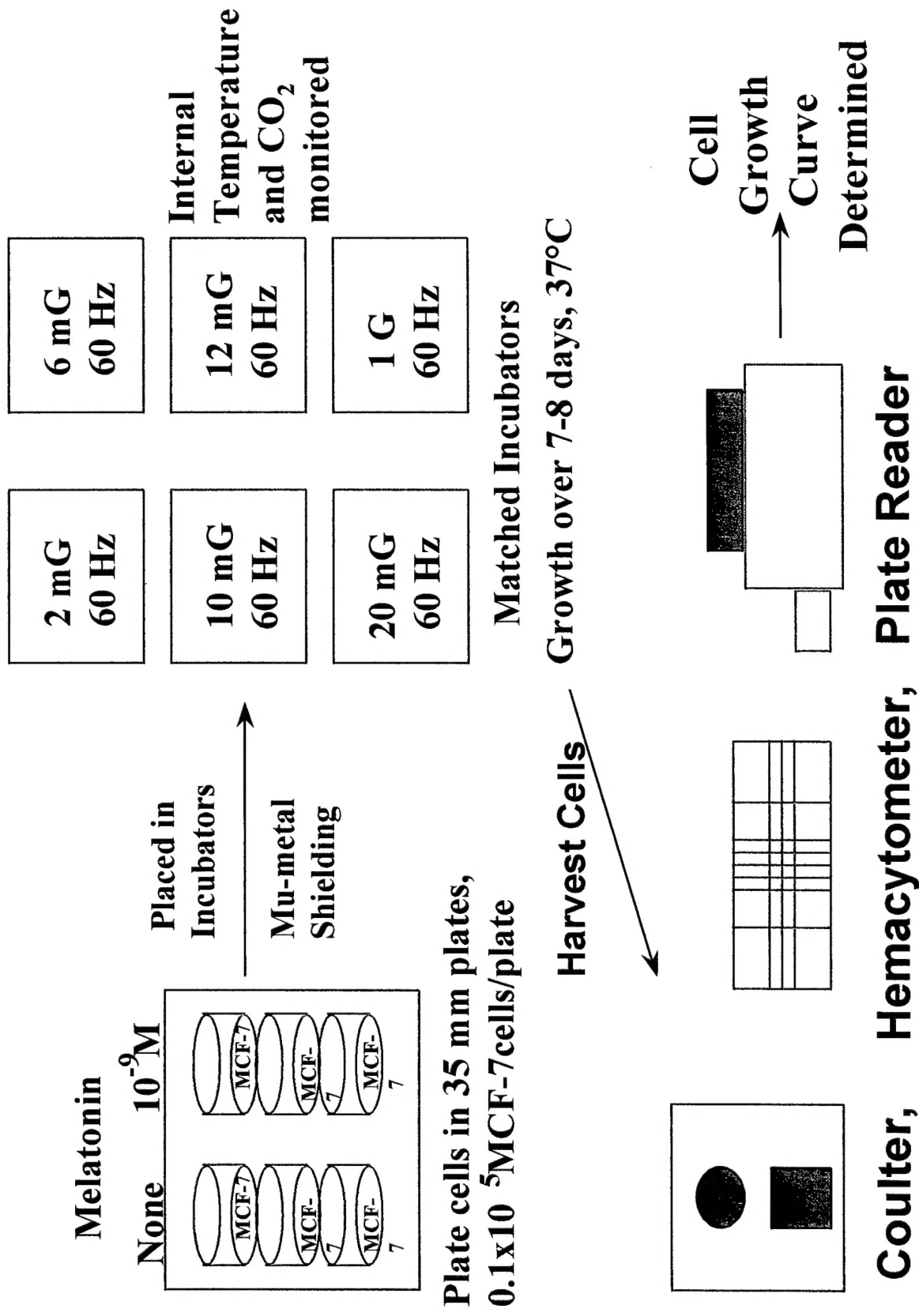


Figure 1

SUMMARY GRAPH:
A Dose Response Is Observed with
an Apparent Threshold at 2 - 12 mGauss

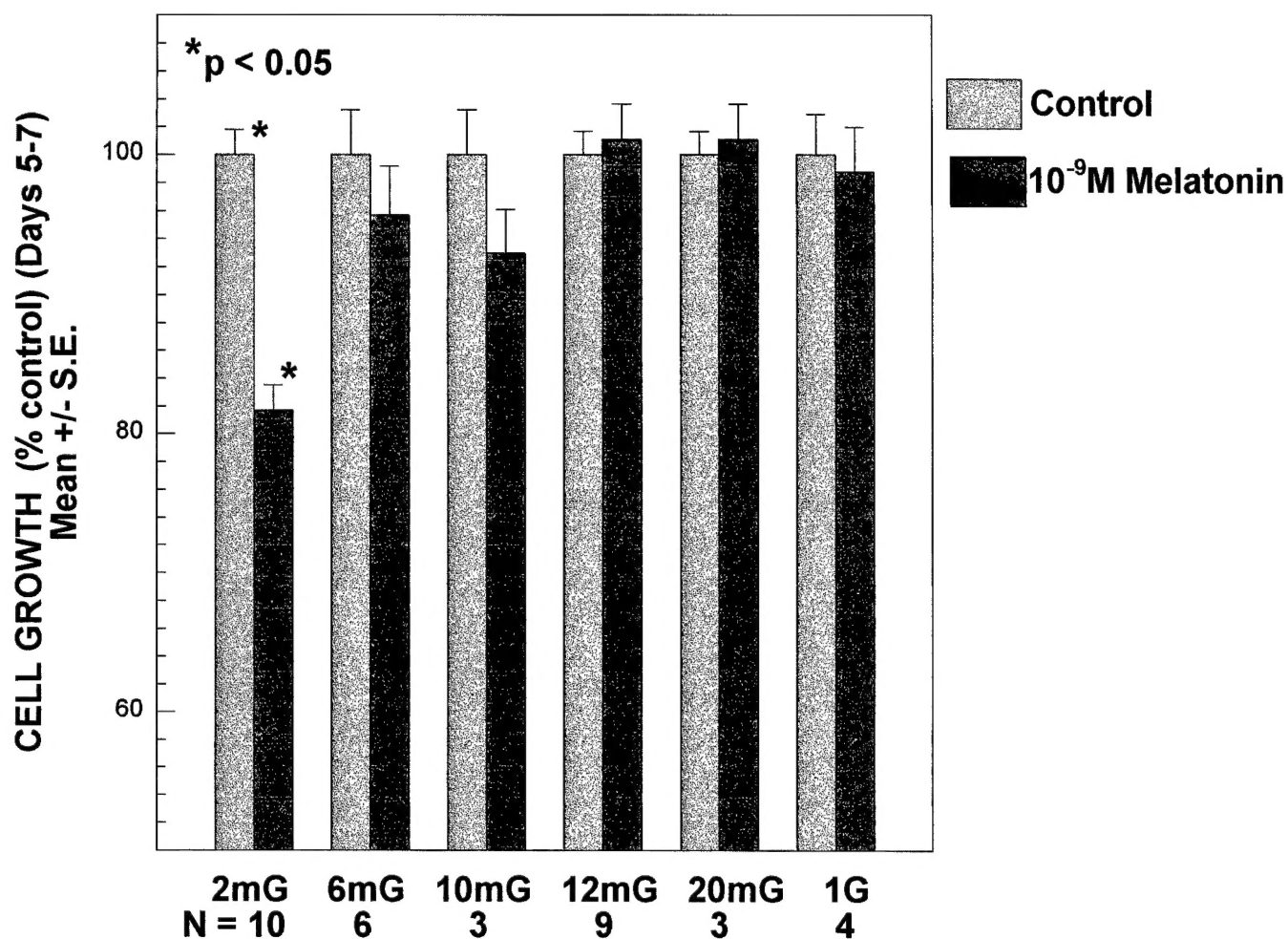


Figure 2

previously observed. These two field treatment groups therefore functioned as quality control parameters in these experiments.

As seen in Figure 2 there is an approximate 20% growth inhibition due to melatonin in a 2 mGauss field. This melatonin effect was inhibited or reduced when cells were exposed to magnetic fields at higher field strengths of 6, 10, 12, 20, and 1000 mGauss in a monotonically increasing manner.

This experimental dose-response data was recently fit to an exponential curve by Dr. Christopher Portier, Chief, Laboratory of Computational Biology and Risk Assessment, NIEHS. He kindly collaborated with us in this effort. We provided him with our experimental data and he fit a monotonically increasing exponential function to our data set. This curve plus the data from our studies is shown in Figure 3. Also, it is important to note that Dr. Portier included Dr. Carl Blackman's data in this modeling. Dr. Blackman has independently replicated our original findings in his laboratory at the EPA. Dr. Richard Luben of the University of California, Riverside has also recently replicated our original findings but we have not yet included his data in this modeling effort. Refer to the reference listing for these citations. Dr. Portier did a test for significance with the exponential function shown in the Figure 3 vs. regression to the mean, and the p value was <0.001. Model prediction was 99.2%. This data and modeling analysis establishes a dose-response relationship.

2. (Y1-Y2) Determine if the exposure metric is either the time-varying magnetic field itself or the induced electric field. Tests based on Faraday's law of current induction will involve rotating the orientation of the magnetic field by 90° to reduce the induced electric field significantly while maintaining the magnetic field intensity.

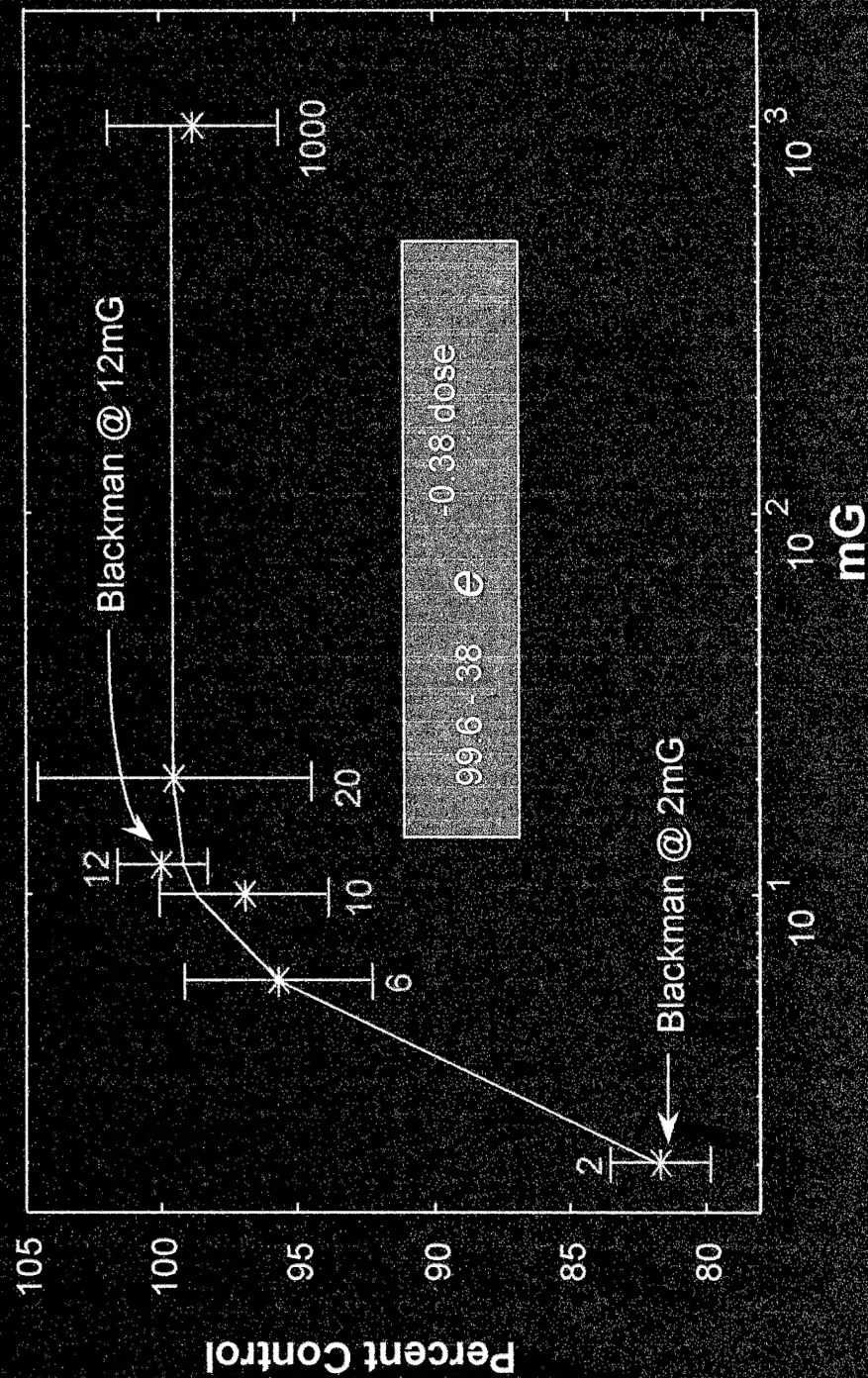
We have established that the magnetic field and not the induced electric field is the operative field metric for blocking melatonin's and tamoxifen's oncostatic action. (Harland & Liburdy, 1997, Bioelectromagnetics, accepted for publication, attached). For experimental details about these experiments please refer to the attached manuscript. These findings are important for several reasons. First, it limits the field interaction to those processes involving a magnetic field. An interaction involving the electric field induced in the cell culture media would likely be operating at the level of the outer cell surface since the electric field does not penetrate beyond the cell membrane at 60 Hz. As a result our follow-on mechanism studies are focusing on identification of possible magnetic field-based interactions. Second, identification of the magnetic field as the operative field metric in our experiments at 12 mGauss may be of some value in the design of future epidemiological studies interested in environmental-level effects of magnetic fields in humans. For example, our dose-response relationship (above) indicates that environmental-level magnetic fields above 2 mGauss might be relevant in exposure assessment, and that exposure-level stratification might be appropriate at 12 mGauss or higher where we see no further increase in blocking of melatonin action by magnetic fields.

3. (Y2-Y3) Determine if there is a frequency dependence. Frequency will be varied between 15 - 300 Hz. This covers a 5-fold range of 60 Hz harmonics.

Although not to be completed in Y2, we have conducted a series of frequency dependency experiments in which it was determined that the field effect for tamoxifen is sensitive to frequency and this was interpreted to mean that relatively slow biological processes on the order of milliseconds or greater (>60 Hz cycle time) are involved in the field transductive step.

The hypothesis we were testing in these studies was developed by Dr. Stefan Engström of the J.L. Pettis Memorial Veterans Administration Medical Center, Loma Linda, CA. The premise

Modeling of Melatonin Dose-Response Data



Dr. C. Portier /1997

is that a relatively fast transductive step would be capable of sensing only an instantaneous value of the applied time-varying 60 Hz magnetic field, and would therefore be relatively frequency independent. In contrast, a relatively slow interaction mechanism, or transductive step, would be sensitive to alterations in frequency, and possibly field orientation as well (S. Engstrom (1997) What is the Time Scale of Magnetic Field Interaction in Biological Systems?, Bioelectromagnetics 18: 244-249.)

We tested whether the 12 mGauss effect on blocking or inhibition of tamoxifen action operated through a relatively slow transductive step by modifying the waveform (and thus frequency) of the applied magnetic field. The original sinusoidal 60 Hz waveform was modified to a rectified version corresponding to 120 Hz frequency. Thus, cell growth was followed for cells treated with a) a 2 mGauss 60Hz magnetic field, or b) a 12 mGauss 60 Hz magnetic field, or c) with a rectified waveform of b). These waveforms are depicted in Figure 4.

A series of seven (7) independent experiments were conducted in which MCF-7 cells treated with tamoxifen or not, and grown in 2 mGauss 60 Hz, 12 mGauss 60 Hz, or 12 mGauss rectified magnetic fields. Growth was determined on day 7 and these data are summarized in Figure 5. We observed that tamoxifen inhibited cell growth by approximately 40% in a 2 mGauss 60 Hz field, and that this was reduced to approximately 20% in a 12 mGauss 60 Hz field. The rectified magnetic field (120 Hz), however, did not block or inhibit tamoxifen's oncostatic action compared to the 2 mGauss 60Hz magnetic field.

We interpret this data to suggest that there exists a biological dynamical complex capable of sensing a 12 mGauss, 60Hz magnetic field, and that this structure has an intrinsic timescale comparable or larger than 17 milliseconds, the period of the applied field.

This information is of importance in narrowing the search for the biological transductive step relevant to this magnetic field interaction. We can rule out a primary transductive step that operates on a relatively fast timescale such as free radical chemistry. There are a number of relatively slow biological-based process that operate at or greater than 17 milliseconds, such as protein folding processes, or ligand binding and conformational alterations in protein 3D structure.

4. (Y3) Determine if there is a threshold for exposure time. We will test if magnetic fields are required during the first 24 hours of hormone/drug interaction with target cells during cell culture. A critical threshold may exist for magnetic field exposure during the cell cycle. Our original exposures were continuous during the eight-day growth period. This is related to Aim 5.

Although this is not a Y1-Y2 item, we have conducted experiments to determine the role that exposure duration may play in a field interaction (Y2 annual report). We have shown that there is a critical exposure time required for the 12 mGauss magnetic field to inhibit tamoxifen's action in MCF-7 cells. Using a "timeshift" protocol described previously in which cells are exposed to 12 mGauss fields for increasing 24 hours increments (one day, two days, etc..) we have observed that at least 3 days of field exposure is required, and this corresponds to approximately once cell cycle of replication. This data suggests that exposure periods of at least one cell cycle may be important and suggests that proteins that regulate cell cycle control points may represent a potentially important target for magnetic fields.

5. (Y4) Determine if the 12 mGauss magnetic field effect is reversible. We will perform a 12 mGauss exposure of MCF-7 cells at the threshold time defined in Aim 4 in the presence of melatonin or tamoxifen, and we will then use these cells to test if they are still

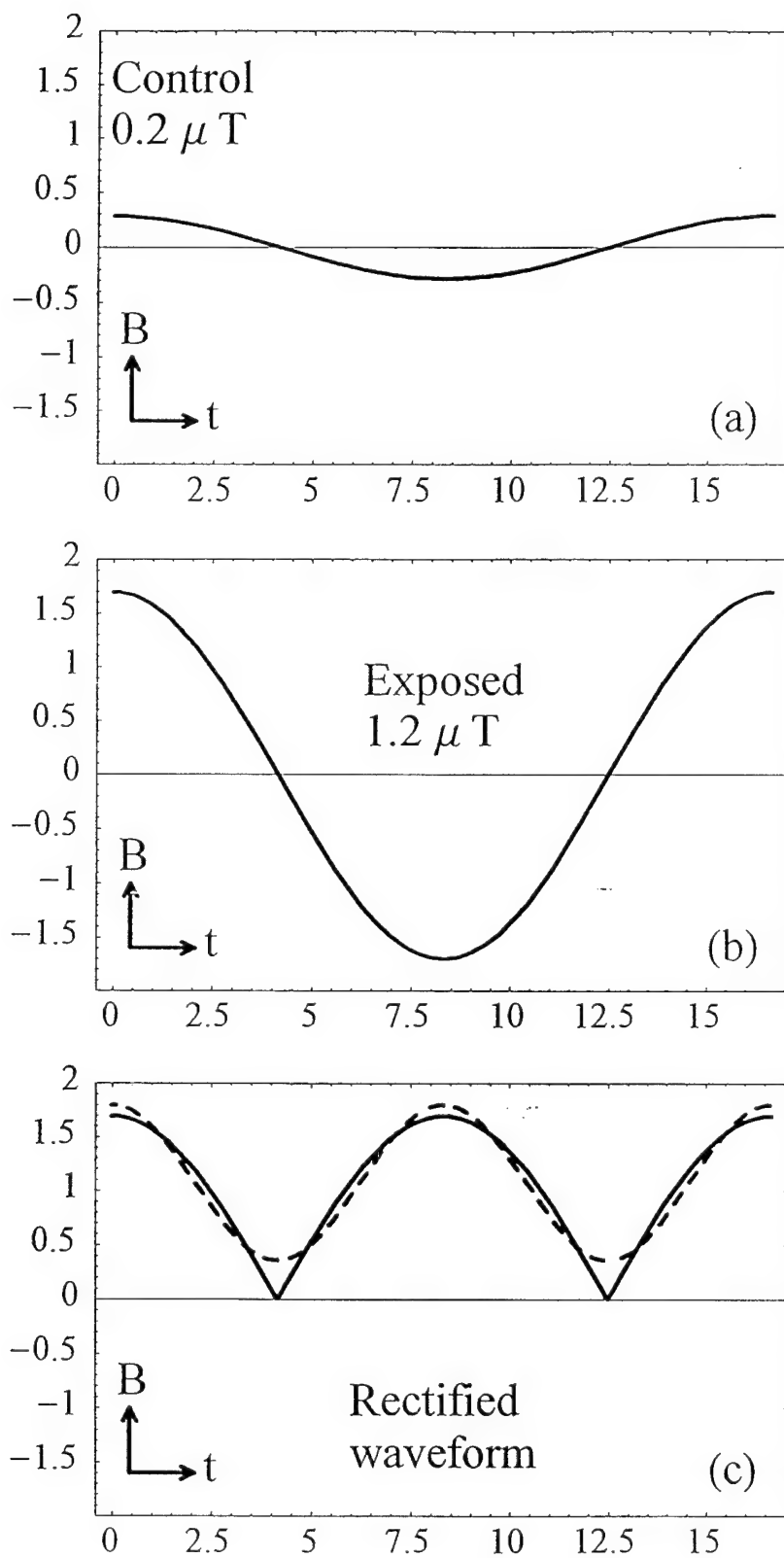


Figure 4

RECTIFIED vs.SINUSOIDAL B FIELD: EVIDENCE FOR A "SLOW" MECHANISM

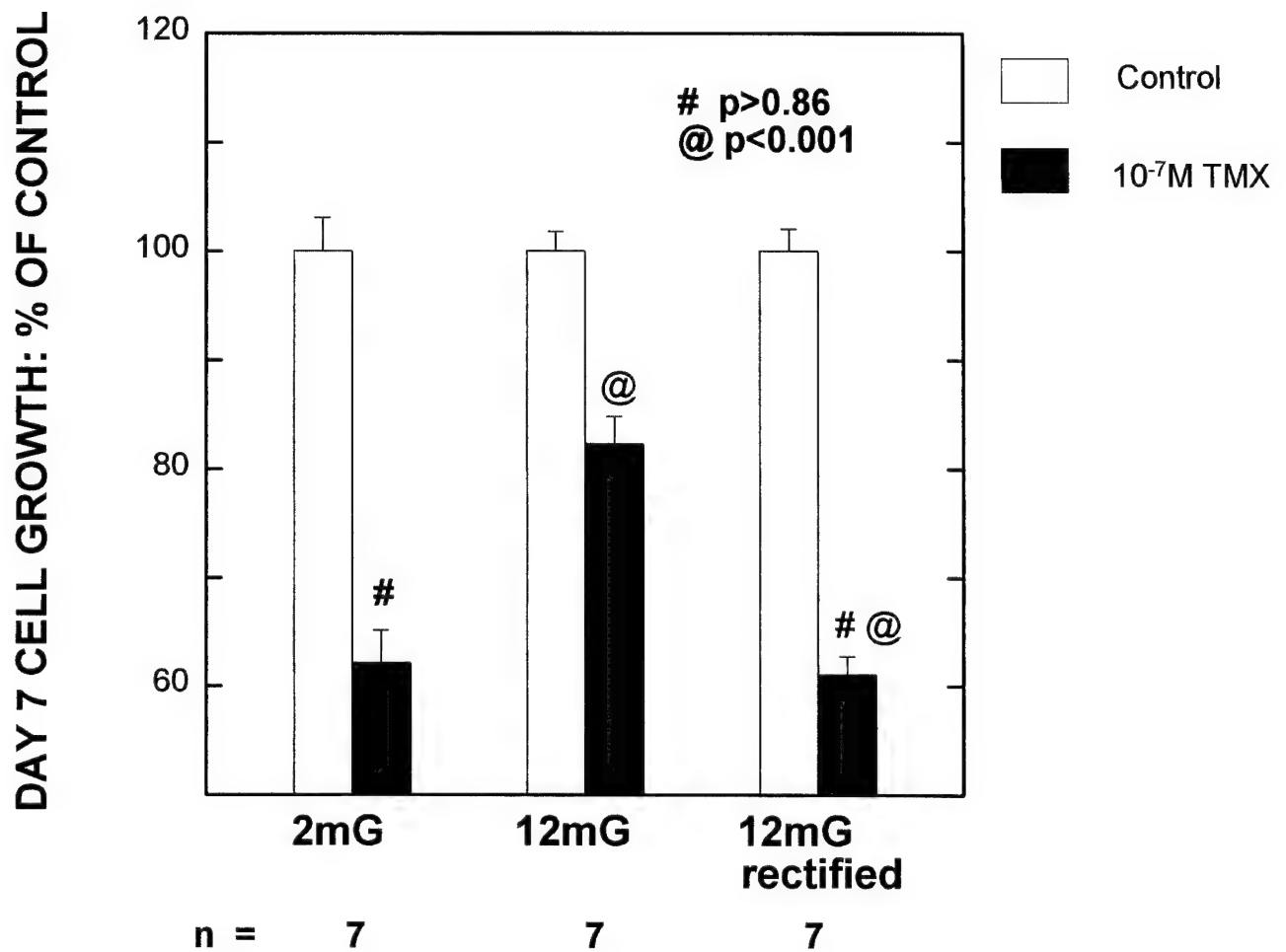


Figure 5

responsive to melatonin or tamoxifen in a second growth curve experiment. The second experiment will employ 2 and 12 mGauss magnetic fields, for comparison.

Not a Y1-Y2 item.

Phase II: Biologically-Based Studies.

6. (Y1-Y2) Determine the dose-response relationship for melatonin and tamoxifen for the 12 mGauss magnetic field effect. We will examine a dose range covering physiological and pharmacological doses of melatonin (10^{-11} to 10^{-5} M) and tamoxifen (10^{-8} to 10^{-6} M), respectively.

We have established that the 12 mGauss field effect is observed for 10^{-11} to 10^{-5} M melatonin, but is restricted to 10^{-7} M tamoxifen, a pharmacological level of this drug that is employed in breast cancer patients. This suggests the possibility of different interaction pathways since the field effect on tamoxifen was not observed at relatively high or low doses of tamoxifen. Refer to the attached articles: Harland & Liburdy, 1997, Bioelectromagnetics, accepted; Liburdy & Harland, 1997, In The Melatonin Hypothesis: Breast Cancer and Use of Electric Power (Eds. R. Stevens, B.W. Wilson, L.E. Anderson), Battelle Press, Columbus, OH.)

7. (Y2) Determine if entry and steady-state levels of melatonin and tamoxifen in MCF-7 cells are altered by the 12 mGauss magnetic field. One way to test this is by following radiolabelled hormone/drug entry into MCF-7 cells.

This aim is being addressed and is not yet completed since we felt it was important to assess possible non-radioactive assays for following hormone/drug entry. We have in part addressed this issue by collaborating with Dr. Steve Yellon in studies designed to assay for melatonin levels in our cell culture media. Dr. Yellon has the capability to assay for melatonin levels in cell culture media in his laboratory in Loma Linda, CA. We have determined in a series of studies with him that concentrations of melatonin in our cell culture media during cell growth experiments are similar in 2 vs. 12 mGauss cell cultures. Thus, the magnetic fields do not apparently alter the availability or utilization of melatonin by cells. We are planning to utilize fluorescence microscopy techniques to assess uptake of melatonin and tamoxifen in single cells.

8. (Y3) Determine if the internal distribution of hormone/drug in the target cell is altered by 12 mGauss magnetic fields. We plan to use quantitative, digital imaging microscopy and antibody-based fluorescent probes at the single-cell level.

Not a Y1-Y2 item.

9. (Y3-Y4) Determine if there is an indirect magnetic field interaction involving signal transduction (ST) which counteracts the growth inhibition action of melatonin and tamoxifen. Some magnetic fields are reported to alter intracellular calcium levels in cells, and intracellular calcium is also linked to ER expression in MCF-7 cells. We plan to assess intracellular calcium at the single-cell level in 2 and 12 mGauss magnetic fields.

Not a Y1-Y2 item.

Experimental Design.

It is important to mention that we have designed and fabricated a special cell culture system for exposing cells in culture to well characterized ELF magnetic fields for these studies. The features of this system have been discussed in previous annual reports, and in the published references listed as citations. Such an exposure system was not in use previously, and we were

prompted to develop such a system since commercial cell culture incubators can generate endogenous AC and DC magnetic fields that vary considerably in space and time.

In these studies we followed an experimental design (discussed above) in which cells are plated out in cell culture plates, treated with melatonin or tamoxifen, and placed into matched, mu-metal shielded incubators corresponding to a desired field strength. We have nine incubators available for such experiments so that cells of the same passage can be used at the same time in a dose-response experiment in incubators set at different field strengths. We feel that this helps to reduce "noise" in comparing data across treatment groups associated with any variation among cell passage number. We always include a set of plates in each incubator that have no drug or hormone so we can compare growth curves across incubators; these should be identical and we use this information as a quality control check-point. We have observed that growth curves are not significantly different for cells that are split and grown simultaneously in matched incubators.

We have also developed a specialized exposure system in which we propagate cells for routine passage in a well-defined 2mG, 60Hz magnetic field. Cells are placed inside of a 4-square Merritt coil and this is housed inside of a mu-metal shielding chamber. This quality control effort insures that our cells are maintained in a uniform, low-level magnetic field for routine passage prior to use in any magnetic field studies.

In addition, we have recently added the capability to measure CO₂ continuously inside of the mu-metal shielding chambers where cells are placed. We employ a small, solid-state, infrared sensor that is placed inside of the exposure coils. Each incubator system is fitted with one of these sensors, which are calibrated with 5% certified CO₂ gas.

Replication Studies.

It is important to mention that our original findings with melatonin have been independently replicated by two laboratories. This is of interest for several reasons. First, since we employed relatively low-intensity, environmental-level, magnetic fields in our studies any bioresponse that is observed at this field level should be verified. Second, our in vitro findings are potentially relevant to human breast cancer risk and independent verification of the original findings would lend credibility to any possible health issues ultimately associated with these in vitro observations.

As mentioned in the annual report for Y2, Dr. Carl Blackman has independently replicated our original melatonin findings. He presented his data at the recent Bioelectromagnetics Society meetings, Victoria, CN, June 9-14, 1996. His abstract citation is included in the reference list. I note that he conducted these studies a) with MCF-7 cells from our laboratory, b) using our detailed laboratory protocols, and c) using the same cell culture media and serum as we use in our laboratory.

Dr. Blackman's results were published in his abstract and are provided below where the mean is times 10⁶ cells per ml. Cells were harvested and counted in a blinded manner.

	<u>Control</u>	<u>Melatonin</u>	<u>Melatonin & MF</u>
B Field	(<2mG)	(<2mG)	(12mG)
mean	1.38	1.15	1.39
S.E.	0.15	0.14	0.14
n	9	9	9

According to the statistical analysis presented in the abstract, the control and melatonin & MF treatments were not significantly different, but both means were significantly larger than the melatonin mean (p<0.001). These findings successfully replicate our original findings.

Recently Dr. Richard Luben of the University of California, Riverside, has also reported that he successfully replicated our original melatonin findings (refer to the reference listing for this citation). He used the same approach as Dr. Blackman in that he a) used MCF-7 cells from our laboratory, b) followed our protocols in conducting the studies, and c) used the identical serum and cell culture media as we employed in our studies. His data is summarized below.

A comparison of experimental conditions across the Blackman, Luben and our laboratory is shown in Table 1. As can be seen the three laboratories used identical biological materials and protocols. There were two differences (shown in red) involving the magnetic fields: a Helmholtz coil was used at EPA instead of a Merritt -type coil, however the magnetic field intensities at EPA were identical to those employed at LBL and Riverside; at Riverside mu-metal shielding was not used, but Dr. Luben verified that the correct AC magnetic field intensities were employed in his studies - also our previous data indicates that DC fields (shielded by the mu-metal chambers) are not a factor in this bioeffect since at LBL similar results were obtained with and without mu-metal shielding (see comment under LBL in Table 1).

A summary of melatonin data from these three laboratories is presented here. Results from our laboratory are summarized in Figure 6. We have observed over sixteen independent experiments (16) to date, that melatonin inhibits MCF-7 cell growth by 25 % in a 2 mGauss field ($p < 0.00001$), and that this was completely blocked in a 12 mGauss field.

Dr. Blackman's data is summarized in Figure 7. At EPA three independent experiments were conducted and they observed that melatonin inhibits MCF-7 cell growth by 17 % in a 2 mGauss field ($p < 0.001$), and this was completely blocked in a 12 mGauss field.

Dr. Luben's data is summarized in Figure 8. At Riverside in three independent experiments they observed that melatonin inhibits MCF-7 cell growth by 13.1 % in a 2 mGauss field ($p = 0.082$), and this was enhanced by 16 % in a 12 mGauss field ($p = 0.0025$). Thus, the 12 mGauss field completely blocked the oncostatic action of melatonin and resulted in a net 29 % enhancement of growth. This is similar to the 25 % growth enhancement observed at LBL, and slightly greater than the 17% growth enhancement observed at 12 mGauss by EPA..

Table 2 presents a composite summary of these findings.

Conclusions.

Below are presented some summary comments about the findings presented in this report and in our previous two annual reports.

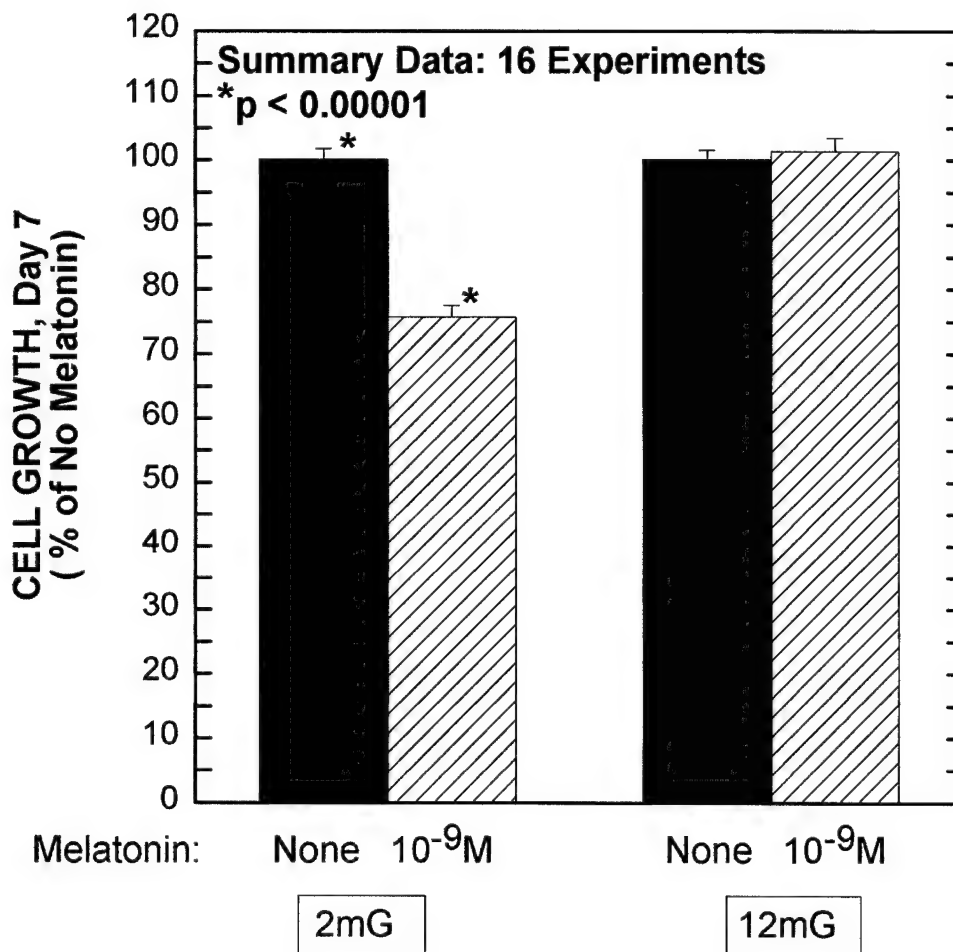
1. Environmental-level EMF magnetic fields alter cell growth in a synergistic manner with a hormone and drug. We have observed and characterized the blocking action of 12mG 60Hz, magnetic fields on the cytostatic function of both melatonin and tamoxifen in MCF-7 cells. To our knowledge this is the first study that has reported environmental-level magnetic fields can modify the growth regulatory actions of hormones or cytostatic drugs on cell growth in vitro.
2. Replication studies confirm our original melatonin findings. Two reports (in abstract form) by two independent laboratories (EPA and the University of California, Riverside) confirm the blocking effect of a 12 mGauss 60 Hz magnetic field on melatonin's oncostatic action in MCF-7 cell in vitro.

Comparison of Experimental Conditions:

	<u>EPA</u>	<u>U.C. Riverside</u>	<u>LBL</u>
<u>B Fields*:</u>	2mG(60Hz)	2mG(60Hz)	2mG(60Hz)
<u>*(rms)</u>	12mG(60Hz)	12mG(60Hz)	12mG(60Hz)
<u>Coil Type:</u>		4-Square Merritt	4-Square Merritt
<u>Mu-Metal</u>			
<u>Shielding:</u>	Yes		No(1993) & Yes(+1993) (Similar Results)
<u>MCF-7</u>			
<u>Cells:</u>	from LBL	from LBL	LBL
<u>Serum:</u>	TCB, Inc. Lot#10786	TCB, Inc. Lot#10786	TCB, Inc. Lot#10786
<u>Protocols:</u>	from LBL	from LBL	LBL
<u>Blinding:</u>	Fields and Melatonin	Fields	Melatonin

Dr. Liburdy's Laboratory Results

**12mG 60 Hz MAGNETIC FIELDS BLOCK
MELATONIN'S NATURAL INHIBITORY ACTION
ON MCF-7 CELL GROWTH**

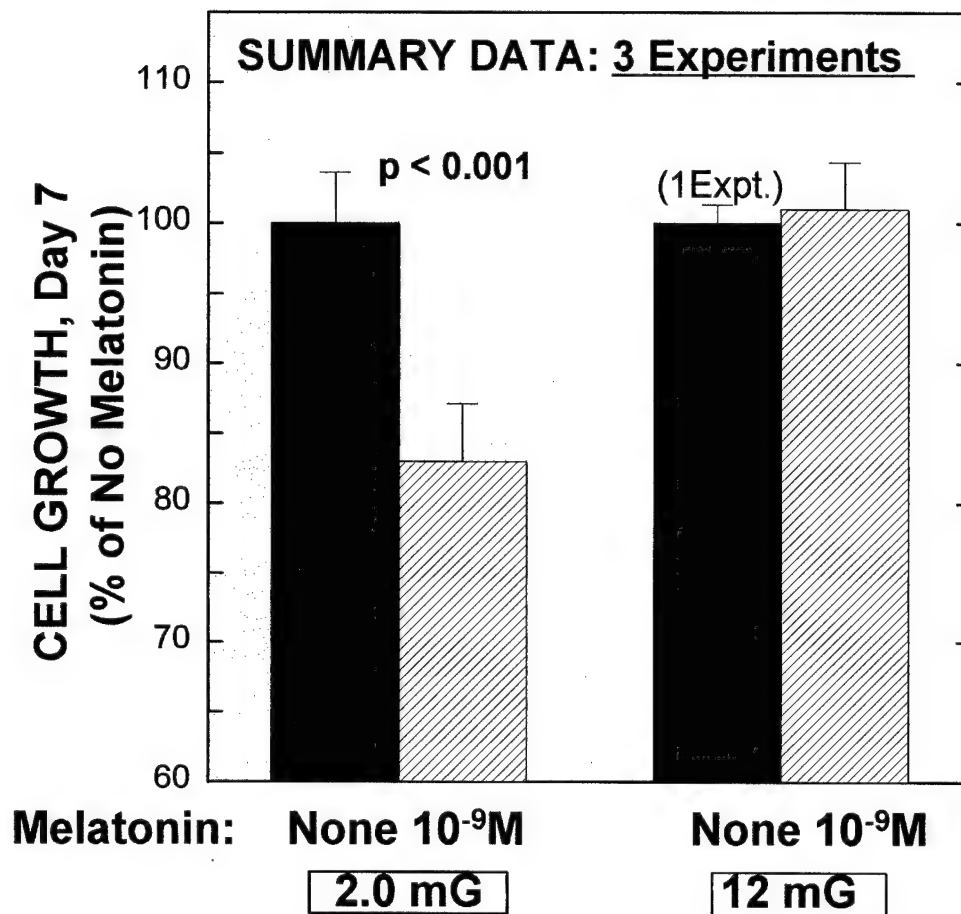


**MELATONIN INHIBITS CELL GROWTH BY 25%
(p<0.00001) IN A 2mG MAGNETIC FIELD.**

**A 12mG FIELD BLOCKS MELATONIN'S ACTION
AND ENHANCES MCF-7 CELL GROWTH.**

**Dr. Blackman's Results Were Presented at the
Bioelectromagnetic Society Meetings, Victoria, CN
June 9 - 14, 1996. Abstract A-1-2.**

**"Independent Replication of the 12mG
Magnetic Field Effect on Melatonin and
MCF-7 Cells in Vitro."**

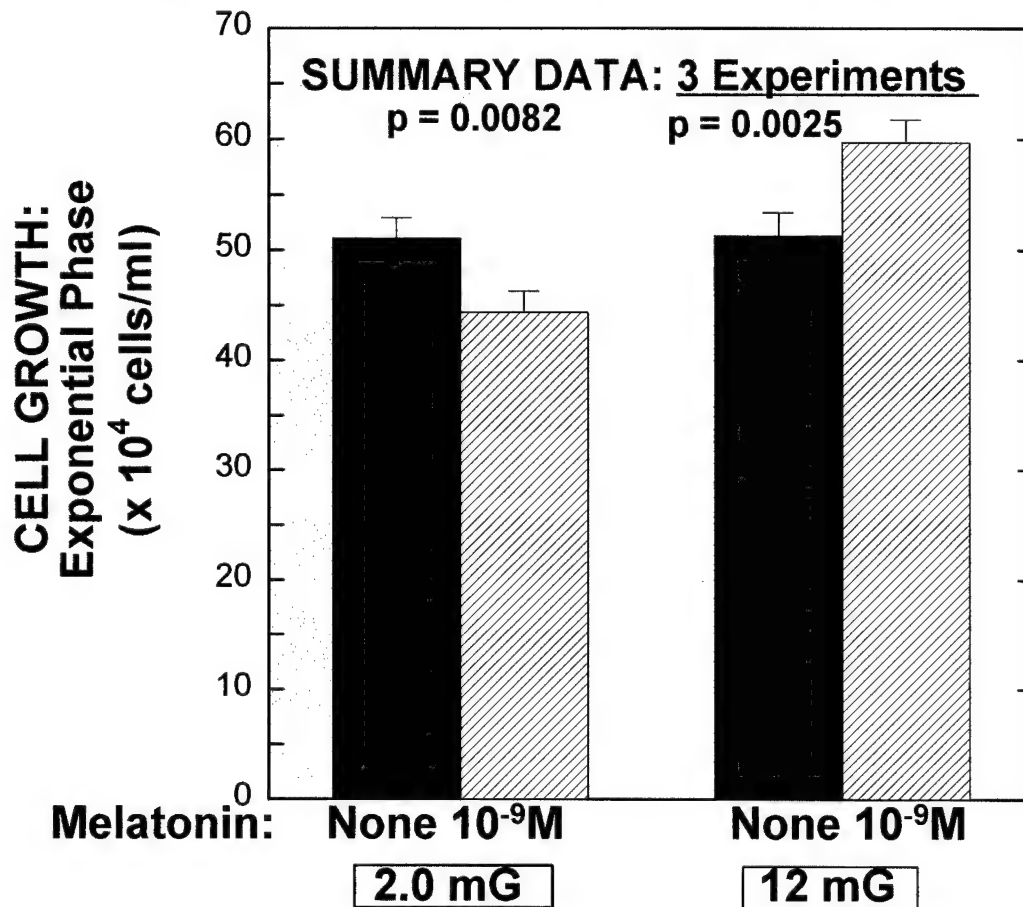


**MELATONIN INHIBITS CELL GROWTH BY 17%
($p < 0.001$) IN A 2mG MAGNETIC FIELD.**

**A 12mG FIELD BLOCKS MELATONIN'S ACTION
AND ENHANCES MCF-7 CELL GROWTH.**

Dr. Luben's Data, D.O.E. Meeting, San Antonio,
TX, November 1996. Abstract A-1.

**"Independent Replication of the 12mG
Magnetic Field Effect on
Melatonin and MCF-7 Cells in Vitro."**



**MELATONIN INHIBITS CELL GROWTH BY 13.1%
(p = 0.0082) IN A 2mG MAGNETIC FIELD.**

**A 12mG FIELD BLOCKS MELATONIN'S ACTION
AND ENHANCES CELL GROWTH BY 16.3%
(p = 0.0025). THIS REPRESENTS A 29% INCREASE
IN CELL GROWTH OVER 2mG MELATONIN-
TREATED CELLS.**

Comparison of Results Across Laboratories:

	<u>EPA</u>	<u>U.C. Riverside</u>	<u>LBL</u>
<u>2mG Field:</u>			
% Melatonin Growth Inhibition	17% p<0.001 n = 3	13% p = 0.0082 n=3	25% p <0.001 n = 16
<u>12mG Field:</u>			
Blocking Effect (Growth Enhancement)	Complete Blocking n = 3	16% Enhancement p = 0.0025 n = 3	Complete Blocking n = 16

3. Dose-response relationship observed. Magnetic field dose-response data establishes that a field dose-response exists between 2 - 12mG for the melatonin findings. This data has been successfully modeled at NIEHS by Dr. C. Portier.

4. Melatonin Action is Blocked at Higher Magnetic Fields. The melatonin blocking effect we observe at 12mG is also observed at the higher field strengths of 20mG and at 1Gauss.

5. Magnetic fields block melatonin and inhibit tamoxifen action at biologically relevant concentrations. Melatonin action is blocked by magnetic fields at physiological concentrations and lower, and tamoxifen action is inhibited at pharmacological doses.

6. Exposure treatment of at least one cell cycle is required. In exposure duration studies it appears that at least two days of treatment to 12mG fields is required for a field blocking effect on tamoxifen action. This suggest a possible role of cell cycle events or regulators in mediating the interaction.

7. Magnetic fields do not alter melatonin concentrations in cell culture media. In studies measuring melatonin concentrations in cell supernatants during 2 vs. 12mG field exposures there is no evidence that melatonin availability to cells (supernatant concentration) is altered. Thus, the 12mG melatonin blocking effect is probably not due to a alteration in melatonin availability in the cell media to MCF-7 cells.

8. Magnetic fields are the operative exposure metric. In studies taking advantage of Faraday's law of current induction, we have established that the operative field metric for both the melatonin and tamoxifen field effect is the magnetic field, not the induced electric field.

9. Frequency dependence. Time scale studies indicate that the transductive step for the magnetic field interaction is consistent with a relatively "slow" biological process that requires 17 milliseconds (one 60 Hz cycle) or greater to "sense" the magnetic field. Candidates are slow processes as protein folding and/or receptor-ligand events. Fast events such as free radical driven processes (e.g., recombination) are ruled out.

References.

Abstracts Presented at National Meetings for Y1 and Y2 (See annual reports for Y1 and Y2).

Research Efforts From our Laboratory.

1. ELF Inhibition of Melatonin's and tamoxifen's Action on MCF-7 Cell Proliferation. J.D. Harland and R.P. Liburdy. Presented at the 1994 Annual Review of Research on Biological Effects of Electric and Magnetic Fields From the Generation, Delivery, and Use of Electricity. Albuquerque, New Mexico, November 6 - 10, 1994. Abstract A-6.

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7. Timescale Hypothesis of Magnetic Field Interaction with Biological Systems: Application to Tamoxifen Model. J.D. Harland, S. Ensgtrom, and R.P. Liburdy. Presented at the 18th Bioelectromagnetics Society meeting held on June 9-14 in Victoria, CN. Abstract P-7A (1996).
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Replication Studies Confirming Our Findings.

1. Independent Replication of the 12mG Magnetic Field Effect on Melatonin and MCF-7 Cells In Vitro. C.F. Blackman, S.G., Benane, D.E. House, J.P. Blanchard. Presented at the 18th Bioelectromagnetics Society meeting held on June 9-14 in Victoria, CN. Abstract A-1-2 (1996).
2. Replication of 12mG EMF Effects on Melatonin Responses of MCF-7 breast Cancer cells In Vitro. R.A. Luben, S. Saraiya & A.P. Morgan. Presented at the 1997 Annual Review of Research on Biological Effects of Electric and Magnetic Fields From the Generation, Delivery, and Use of Electricity. San Antonio, TX, November 19-21, 1996. Abstract A-1.

Appendix

1. Magnetic Fields, Melatonin, Tamoxifen, and Human Breast Cancer Cell Growth (1997) R.P. Liburdy & J.D. Harland. *In* The Melatonin Hypothesis: Breast Cancer and Use of Electric Power (Eds. R. Stevens, B.W. Wilson, L.E. Anderson), Battelle Press, Columbus, OH.) pg. 669-700.

2. Environmental Magnetic Fields Inhibit the Antiproliferative Action of Tamoxifen and Melatonin in a human Breast Cancer Cell Line. J.D. Harland & R.P. Liburdy (1997). In Press, *Bioelectromagnetics*.

3. Independent Replication of the 12mG Magnetic Field Effect on Melatonin and MCF-7 Cells In Vitro. C.F. Blackman, S.G., Benane, D.E. House, J.P. Blanchard. Presented at the 18th Bioelectromagnetics Society meeting held on June 9-14 in Victoria, CN. Abstract A-1-2 (1996).

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THE MELATONIN HYPOTHESIS

Breast Cancer
and Use of
Electric Power

EDITED BY
Richard G. Stevens
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22 Magnetic Fields, Melatonin, Tamoxifen, and Human Breast Cancer Cell Growth

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INTRODUCTION

This chapter reviews experimental evidence obtained in our laboratory indicating that environmental-level magnetic fields [12 mG ($\approx 1.2 \mu\text{T}$), 60 Hz] can interfere with the growth-regulating actions of the hormone melatonin and the cancer drug tamoxifen in cell culture.

In studies in our laboratory over the past several years, we have observed that 12-mG, 60-Hz magnetic fields can act to block the growth inhibition of physiological levels (10^{-9} M) of melatonin on MCF-7 human breast cancer cells in vitro. Composite data over this period suggest that the static DC magnetic field is apparently not critical for the blocking effect of such fields on melatonin action. Interestingly, the same 12-mG, 60-Hz magnetic fields appear to inhibit the cytostatic action of pharmacological doses (10^{-7} M) of tamoxifen. Regarding the magnetic field metric for this interaction, the 12-mG magnetic field (B), not the associated induced electric field (E) due to Faraday's Law of Current Induction, is associated with the field effect. Thus, the magnetic field appears to be the operative exposure metric.

Performing cell culture studies requires carefully controlling the magnetic field inside of cell culture incubators. A stray magnetic field can significantly confound the reproducibility of data, both within and across laboratories. Therefore, we developed a special exposure system to generate uniform, 60-Hz magnetic fields using four-square Merritt coils enclosed in specialized mu-metal chambers to eliminate stray, time-varying AC and static DC magnetic fields.

Recently, an independent replication of the 12-mG inhibition of melatonin action has been reported; this study is discussed herein. It is of mechanistic interest that, according to our data, a 12-mG magnetic field inhibits tamoxifen action, because this raises the possibility that the estrogen receptor may be a possible biochemical site for this field interaction. Further studies of the type described here, employing a well-characterized exposure system and cellular model system, will shed light on magnetic field interactions with biological systems.

BACKGROUND

Experimental evidence from cellular, animal, and human laboratory studies exists which suggests that environmental-level electric and magnetic fields (EMF) might represent a potential risk factor for

human breast cancer via a field effect that depresses or time-shifts melatonin secretion into the blood (Pool 1990; Wilson et al. 1981, 1983, 1986; Welker et al. 1983; Yellon 1993; Graham et al. 1993, 1994; Kato et al. 1994a, b; Löscher et al. 1994). Also, some recent epidemiologic studies have raised the possibility that EMF exposure may be a potential risk factor for breast cancer (Tynes and Anderson 1990; Matanoski et al. 1991; Demers et al. 1991; Loomis et al. 1994).

That low-frequency, time-varying EMF may depress or time-shift melatonin secretion has been reported by several investigators. This effect was first reported by B. Wilson in rats (Wilson et al. 1981, 1983, 1986), and has since been observed in cultured pinealocytes (Welker et al. 1983), hamsters (Yellon 1993), rats (Kato et al. 1994a; Löscher et al. 1994), and in some human volunteers exposed to 200-mG, 60-Hz magnetic fields at night (Graham et al. 1993, 1994). Suppression of melatonin has been ascribed to an effect of these fields on the pineal gland, which produces melatonin. Melatonin has a variety of biological functions, including circadian rhythm control, endocrine function inhibition, immune function enhancement, and oncostatic properties (Maestroni 1993; Reiter in press; Guerrero and Reiter 1992). Relevant to the latter is the observation that melatonin is reported to inhibit 7,12-dimethylbenz(a)anthracene (DMBA)-induced rat mammary gland carcinogenesis (Subramanian and Kothari 1991). Consistent with an in vivo model of interaction involving melatonin, recent animal studies have reported that magnetic fields can enhance DMBA breast cancer growth in rats (Mevisen et al. 1993; Löscher et al. 1993). In addition, D. Blask and coworkers have reported that melatonin inhibits the in vitro growth of MCF-7 cells, an estrogen receptor-positive human mammary tumor line, further supporting the oncostatic properties of melatonin (Hill and Blask 1988; Cos and Blask 1990; Cos et al. 1991). Other groups have subsequently reported on the oncostatic action of melatonin in MCF-7 cells (Molis et al. 1994 and references therein, 1995; Cos and Sanchez-Barceló 1994, 1995; Crespo et al. 1994). One possible mechanism envisioned for EMF effects, then, is a field-related depression of melatonin, leading to increased breast cancer risk (Stevens et al. 1992).

In our laboratory, we have addressed the possibility of a more direct, but complementary mechanism for an effect of EMF on breast cancer; namely, the interaction of the field with the target cell to alter

melatonin's biological action. In support of this hypothesis, we recently reported that environmental-level, 12-mG, 60-Hz magnetic fields block melatonin's natural growth-inhibitory action on MCF-7 cells in culture, while having no effect on untreated cell growth (Liburdy et al. 1993a, b). These two modes of field action—depression of pineal gland melatonin secretion and blocking of melatonin's action on target cells—raise the possibility of a synergistic effect *in vivo*.

It is significant that our melatonin finding recently has been independently replicated (Blackman et al. 1996). Replication studies are difficult to execute and require commitment, careful attention to detail, and should follow three basic rules:

1. In cellular experiments, the same cells should be obtained from the originating laboratory and the same serum employed.
2. The same protocols should be followed, and these protocols should be well-defined and made available from the originating laboratory.
3. Similar or identical exposure systems as used in the originating laboratory should be employed.

Although there were slight variations from our methods in the protocols employed in the replication study reported by C. Blackman of the U.S. Environmental Protection Agency (EPA), such as using a Helmholtz coil instead of a four-square Merritt coil, there was apparently enough detail in the protocols to identify the important variables in the MCF-7 experiments. This study, we believe, is the first such true replication of a key magnetic field-induced bioeffect.

To conduct the studies reviewed here, we designed and implemented a special exposure system that (1) generates a uniform magnetic field for exposure of cells in culture, and (2) employs a mu-metal shielding chamber to eliminate the confounding presence of spurious, time-varying AC and static DC magnetic fields generated by commercial incubators (Liburdy et al. 1993b; Liburdy 1994a, 1995). This exposure system (operating at 2 mG, 60 Hz) is used in our laboratory to grow and maintain MCF-7 cells prior to their use in higher-field exposures. This process assures us that the past EMF history of the cells is well-characterized before use in experiments.

In considering a possible mechanism for magnetic field effects, we asked whether such fields decrease the growth-inhibitory action of the drug tamoxifen. Tamoxifen is the most widely used anti-estrogen therapy for the control of breast cancer, and, importantly, is known to bind to and probably act via the estrogen receptor (Coezy et al. 1982; Martin et al. 1988). Our observation that a 12-mG, 60-Hz magnetic field can inhibit tamoxifen action in culture raises the possibility of estrogen receptor involvement in this field interaction. Such an observation opens an avenue for future study that may shed light on possible biochemical sites with which magnetic fields may interact.

MATERIALS AND METHODS

Cells, Hormones, and Drugs

The MCF-7 cell is an epithelial-like cell derived from the pleural effusion of a mammary adenocarcinoma (ATCC HTB-22) (Soule et al. 1973). Melatonin-sensitive MCF-7 cells at passage #18 were a generous gift of Dr. David Blask of the Mary Imogene Bassett Hospital Research Institute, Cooperstown, New York. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; 1.0 g/L glucose, #D8788, Sigma Chemical Co., St. Louis, Missouri) supplemented with 10% fetal bovine serum (#101, Lot #10786, Tissue Culture Biologicals, Tulare, California), penicillin (200 units/mL), and streptomycin (100 μ g/mL) (penicillin/streptomycin: University of California-San Francisco Cell Culture Facility). Use of high-glucose DMEM or some serum lots was observed to diminish melatonin sensitivity. Cells were grown as a monolayer at 37°C in a humid atmosphere, with 5% CO₂. Melatonin (N-acetyl-5-methoxytryptamine; #M5250) and tamoxifen (#T9262) were purchased from Sigma Chemical Co. Melatonin and tamoxifen solutions were prepared before use by dissolving crystals in minimum ethanol, followed by dilution in media.

Magnetic Field Exposure System

The cell culture exposure system we developed and use in our laboratory has been described previously (Liburdy et al. 1993b; Liburdy 1994a, 1995). To address the engineering issue of contaminating mag-

netic fields present inside commercial cell culture incubators, we designed an exposure system that generates a uniform magnetic field environment free of stray, time-varying magnetic or electric fields associated with operating the incubator. Special features are: (1) a perforated Plexiglas platform table, within (2) a double-wound, four-coil Merritt exposure system (plastic frame wound with double-wrap, bifilar cable, in the Merritt's turn ratio of 26/11/11/26), placed inside (3) a ventilated mu-metal chamber to eliminate extraneous time-varying and static magnetic fields, within (4) a water-jacketed Queue incubator (Queue Systems Inc., Parkersburg, West Virginia, Model 2710), maintained at $37^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$. We chose the four-square Merritt coil, because it provides the largest uniform exposure volume (Merritt et al. 1983; Kirschvink 1992b) (Fig. 1). We placed these exposure coils inside a mu-metal chamber (Fig. 2). This chamber (Magnetic Shield Corp., Perfection Mica Co., Bensenville, Illinois) has ventilation holes on the top and bottom, and is constructed of nickel and trace metals. This design allows effective shielding of cells inside from spurious magnetic fields generated during operation of the incubator so that the interior static magnetic field levels are approximately > 10 mG, and AC magnetic field levels are essentially not measurable at extremely low frequency (ELF) using our dosimetry probes (see next paragraph). Signal generators were used to drive the coils (Dynascan Corp., Chicago, Illinois; B & K Precision Model 3020); coils were shielded with aluminum or copper foil to eliminate the electric field associated with each current-carrying coil. Figure 3 shows the complete exposure system in place inside a commercial incubator.

Field dosimetry was performed using a commercially available fluxgate magnetometer (Hewlett Packard Model 428B fluxgate meter, Cupertino, California), calibrated by the Magnetic Field Measurements Group at Lawrence Berkeley Laboratory, or by us in our laboratory using a calibration coil we designed. Field readings were taken before, during, and after most experiments, and yielded values within approximately $\pm 5\%$. We also have performed measurements using a Multiwave II System (Electric Research and Management Inc., State College, Pennsylvania); cross-calibration of our Hewlett Packard fluxgate probes with a Multiwave II Bartington probe gave values within approximately $\pm 15\%$. Merritt coils generated very uniform field

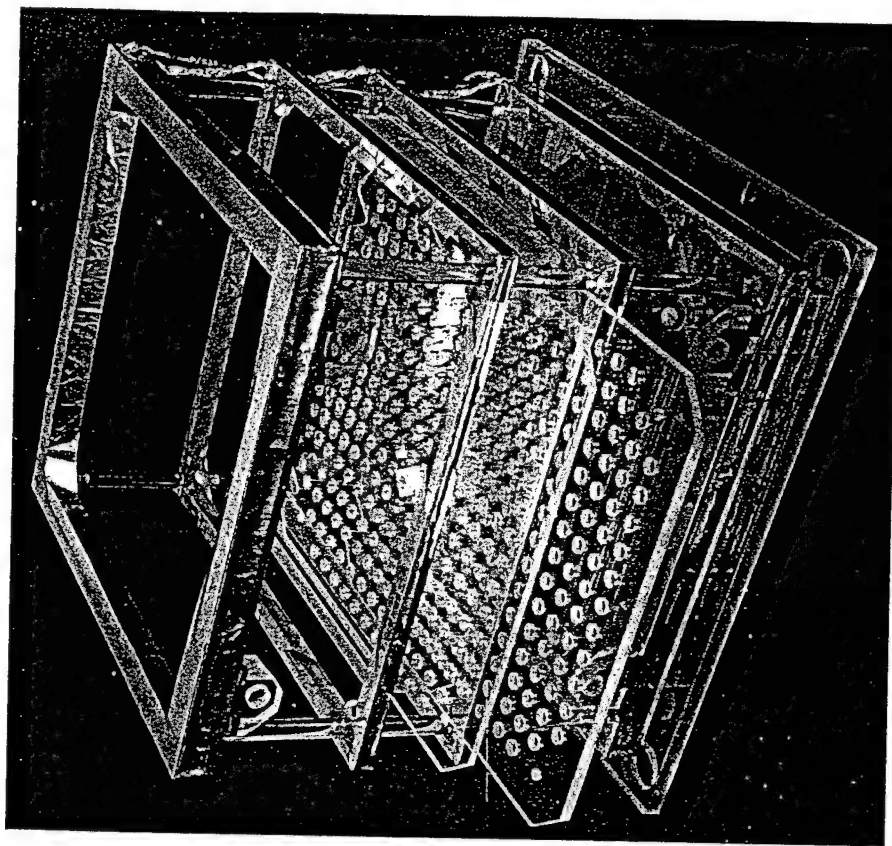


FIG. 1. Cell culture exposure coil. A Plexiglas frame was constructed to accommodate four square coils that are wound in a specific turn ratio, and that are separated according to a special relationship described by Merritt et al. (1983). The frame is essentially a cube with an edge length of approximately 35 cm. The volume between the two central coils is associated with a very uniform ($< 2\%$ variation) magnetic field when the coil is energized (Liburdy 1995; Kirschvink 1992b). In the design we employed, the coil frame can be physically rotated about the platform for holding the cell culture plates by 90° , so as to re-orient the magnetic field vector from a perpendicular to a parallel direction with respect to the cell culture plate. Refer to Figure 8 and text.

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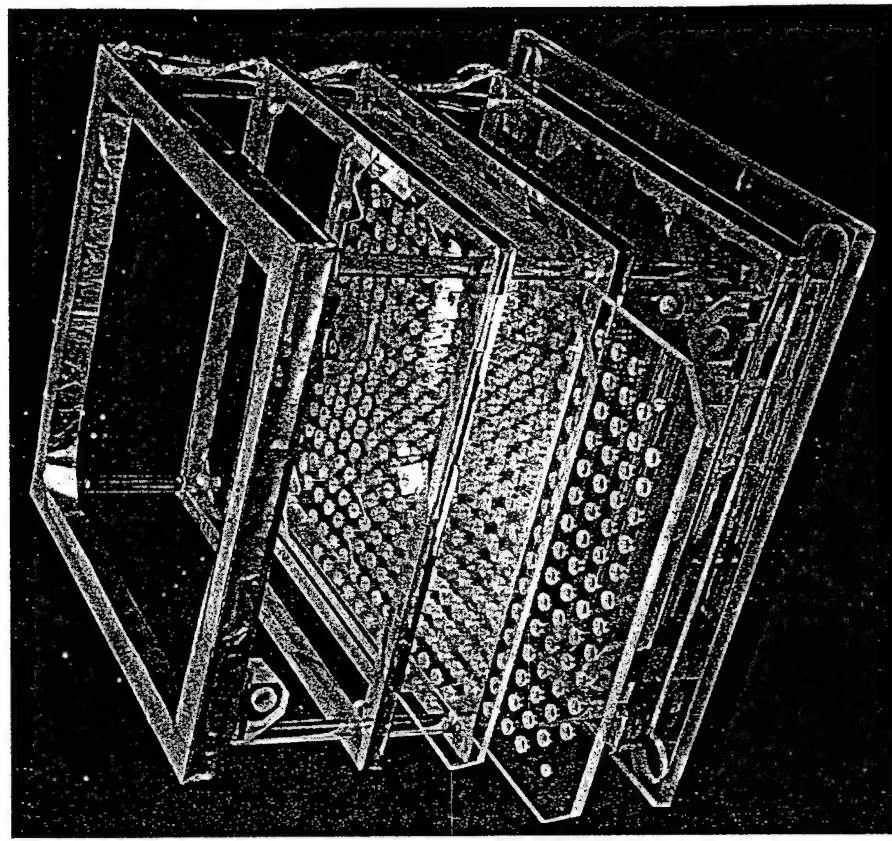


Fig. 1. Cell culture exposure coil. A Plexiglas frame was constructed to accommodate four square coils that are wound in a specific turn ratio, and that are separated according to a special relationship described by Merritt et al. (1983). The frame is essentially a cube with an edge length of approximately 35 cm. The volume between the two central coils is associated with a very uniform ($< 2\%$ variation) magnetic field when the coil is energized (Liburdy 1995; Kirschvink 1992b). In the design we employed, the coil frame can be physically rotated about the platform for holding the cell culture plates by 90° , so as to re-orient the magnetic field vector from a perpendicular to a parallel direction with respect to the cell culture plate. Refer to Figure 8 and text.

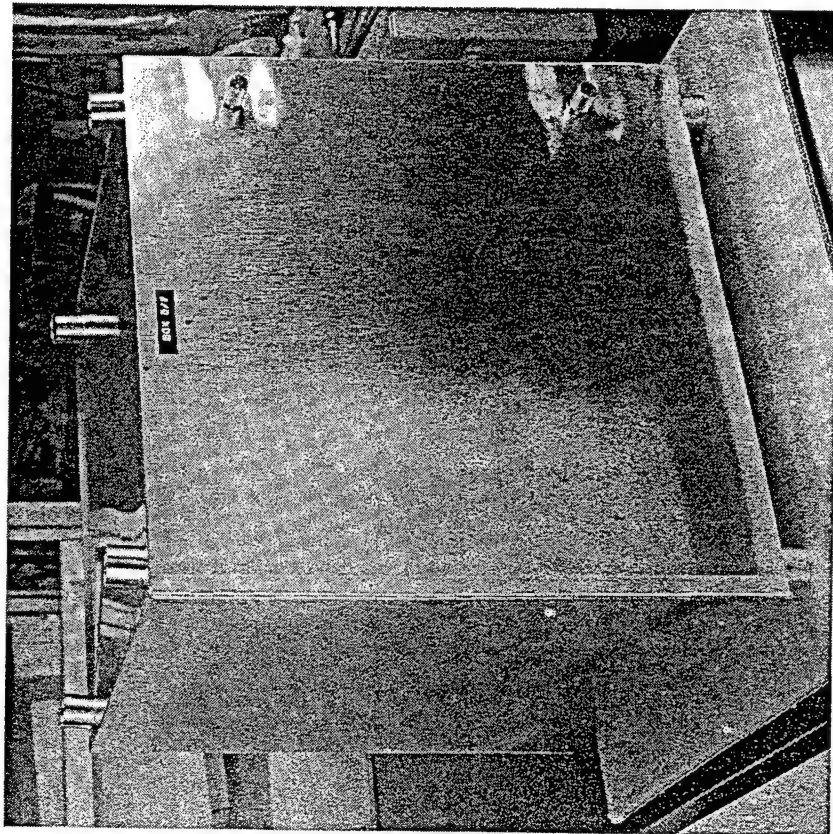


FIG. 2. Mu-metal chamber. This chamber is a cube that was constructed from Co-Netic™ AA alloy (~ 80% nickel) that is approximately 1.016 mm thick with an edge length of approximately 40 cm (Magnetic Shield Corp., Bensenville, Illinois). The chamber was butt-seam welded and hydrogen annealed after fabrication, and has a hinged door that can be secured to the chamber. Four ventilation holes on the top and bottom are approximately 2.54 cm in diameter, and have an extension tube of 2.45 cm in length; these holes are located in the corners of the cube form.

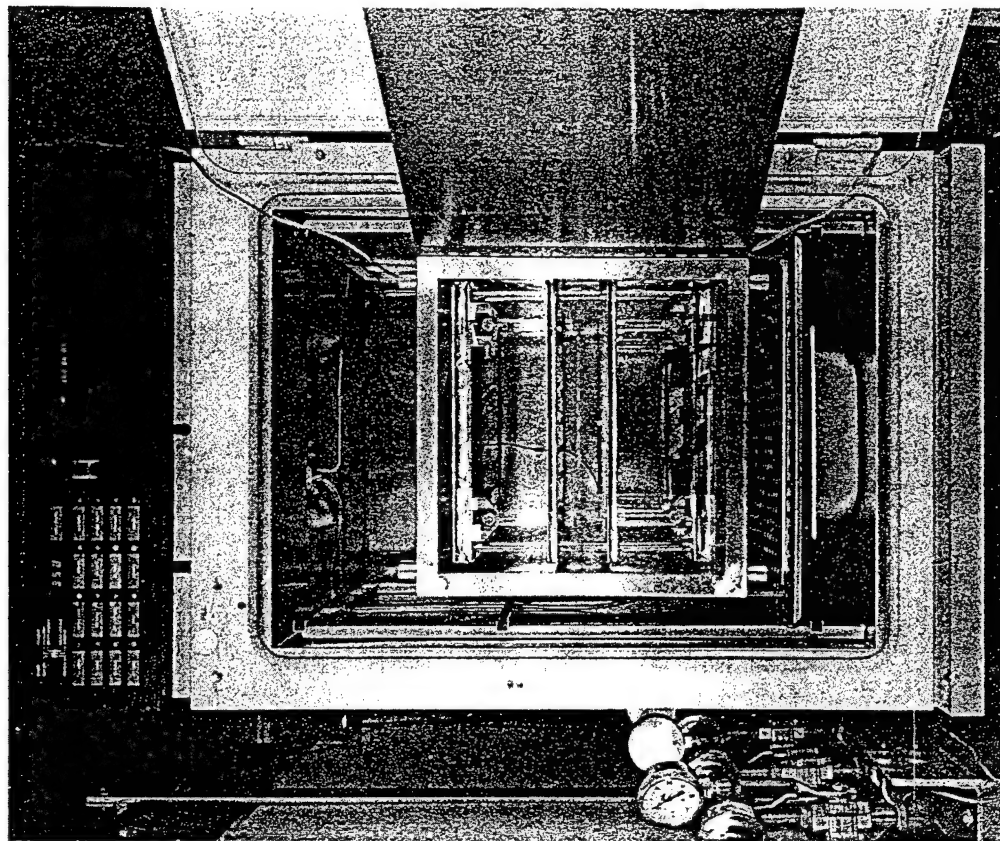


FIG. 3. Cell culture exposure system. Shown is the combination of the four-square Merritt coil and the mu-metal chamber, which are both placed inside a commercial cell culture incubator. Also shown is a thermistor temperature probe, which is threaded through one of the ventilation holes into the mu-metal chamber and placed inside the chamber at the position where cell culture plates are typically located.

values (Merritt et al. 1983; Kirschvink 1992b) over the central volume where our cells were placed; static DC fields were reduced to approximately 0.1 mG by the mu-metal shields. We performed 2-mG and 12-mG field exposures simultaneously using three identical exposure systems. This approach permitted experiments to be conducted on same-passage cells that were exposed to (1) 2-mG magnetic fields oriented perpendicular to Petri dishes, (2) 12-mG magnetic fields oriented parallel to Petri dishes, and (3) 12-mG magnetic fields oriented of cell growth. Temperature was monitored continuously and recorded daily, using thermistor probes (YSI Inc., Yellow Springs, Ohio) placed inside the mu-metal chambers and located next to the cell culture plate area.

Cell Culture Techniques

We passaged MCF-7 cells on Falcon 60-mm plates (Becton Dickinson, New Jersey). For drug/hormone sensitivity assays, cells were harvested in 0.2% ethylenediaminetetraacetic acid (EDTA) phosphate buffer (2 g/L $\text{Na}_2\text{-EDTA}$, 8 g/L NaCl , 0.2 g/L KH_2PO_4 , 1.15 g/L Na_2HPO_4), separated into a single-cell preparation by passing three times through a 25-gauge needle, and seeded at 0.1×10^5 cells/35-mm dish in 1.5 mL of media. By 4 hours, the cells had attached and media was changed, with or without drug/hormone additions. Cell growth was followed up to days 5–8. Cells were maintained continuously in original culture media and exposure conditions until counting. On counting days, cells were detached with 0.2 mL trypsin solution at 37°C (0.50 g/L trypsin, 0.5 g/L EDTA, 1.0 g/L glucose, and 0.58 g/L NaHCO_3), diluted with 0.8 mL PBS, and counted by hemacytometer. Three plates for each treatment category were counted per counting day.

Melatonin sensitivity of MCF-7 cells is affected by several factors, including source of fetal bovine serum, schedule of media change, MCF-7 cell passage number, cell density at time of harvesting, cell seeding density, and thoroughness of single-cell preparation at time of seeding. Although several groups have reported that melatonin exhibits oncostatic properties in MCF-7 cells (Molis et al. 1994 and references therein; Cos and Sanchez-Barceló 1994, 1995; Crespo et

al. 1994), we note that one group of investigators has reported that an MCF-7 subclone studied in their laboratory failed to respond to melatonin (L'Hermite-Baleriaux and de Launoit 1992). We have prepared extensive written protocols for our procedures that are available upon request.

Statistical Analyses

Data shown here were tested for statistical significance using the SigmaPlot Student t-test (Jandel Corporation, Corte Madera, California). The multifactor analysis of variance program in Statgraphics (Manugistics Inc., Rockville, Maryland) also was used to analyze data in Figure 5 across melatonin doses. Error bars in the figures represent the standard error of the mean.

RESULTS

Inhibition of Melatonin Action by a 12-mG, 60-Hz Magnetic Field

We have observed that exposure to a 12-mG, 60-Hz field and a 135- to 145-mG, DC magnetic field blocks the growth-inhibitory action of melatonin on MCF-7 breast cancer cells (Liburdy et al. 1993b). In Figure 4, experimental data are presented showing the effect of 60-Hz, 2-mG or 12-mG magnetic fields on melatonin's growth inhibition of MCF-7 cells using mu-metal shielded cultures. Effect of the field is shown for the day (5, 6, or 7) of maximum melatonin effect. Across seven experiments, treatment with the physiological dose of 10^{-9} M melatonin in 2-mG magnetic fields resulted in an approximate 26% inhibition of MCF-7 cell growth ($p < 0.00001$; 100% is defined as growth in the absence of hormone). Exposure to 12-mG magnetic fields, however, blocked melatonin's action. This result confirms our previously reported observations (Liburdy et al. 1993a, b), and also suggests that static magnetic fields (≤ 145 mG) may not play a role in this effect. We note that two preliminary reports in abstract form indicate results consistent with these 60-Hz effects (Blask et al. 1993a, b). More importantly, these findings have been independently replicated at the EPA (Blackman et al. 1996).

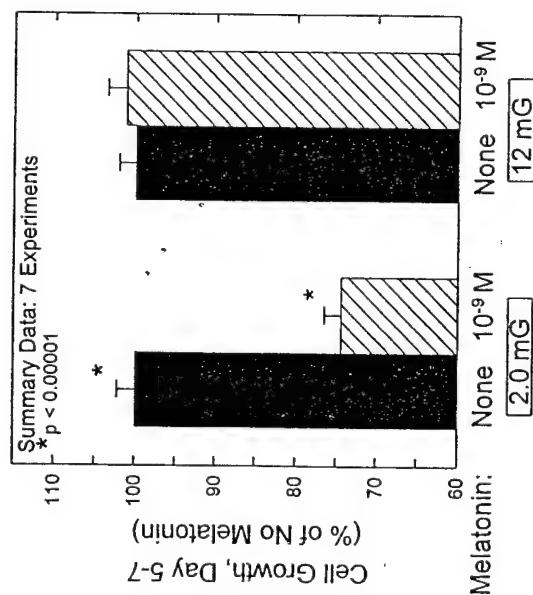


Fig. 4. Comparison of melatonin inhibition of MCF-7 cell growth in the presence of 2-mG or 12-mG fields; summary data from seven experiments. Cells were seeded at 0.1×10^5 /plate and grown in media containing no melatonin or 10^{-9} M melatonin in an incubator with a background 60-Hz magnetic field of 2 mG, or a 60-Hz magnetic field of 12 mG. In all experiments, cultures were mu-metal shielded (Liburdy 1994b, 1995). In a 2-mG background field, melatonin exhibited on average 26% inhibition of MCF-7 cell growth on days 5-7 (see text); in a 12-mG field, melatonin had no significant effect on cell growth.

In his studies, Dr. Blackman received MCF-7 cells from our laboratory, as well as extensive written protocols describing our laboratory procedures. Dr. Blackman conducted three experiments in which he measured cell growth (cell counts) on day 7 for cells in three categories, as detailed in Table 1. This data represents an independent replication of our earlier findings (Liburdy et al. 1993b). In follow-on control studies, Dr. Blackman evaluated cell growth of MCF-7 cells in the absence of melatonin in a 12-mG versus 2-mG, 60-Hz magnetic field (using mu-metal shielded chambers). This experiment addressed the question of whether the 12-mG field may influence MCF-7 cell growth, per se. When Dr. Blackman assessed the growth of five independent plates of

TABLE 1
Cell Growth in Replicate Studies by Blackman et al. (1996)

	Control	Melatonin	Melatonin & B field
B field	(< 2 mG)	(< 2 mG)	(12 mG)
Mean ^a	1.38 ^b	1.15 ^b	1.39
SE	0.15	0.14	0.14
n	9	9	9

^a The mean is expressed $\times 10^5$ cells.

^b The means were significantly different ($p < 0.001$).

cells cultured in the absence of melatonin at < 2 mG or 12 mG, he observed no statistically significant difference between the magnetic field-treated cell cohorts ($p = 0.713$). Thus, the magnetic field by itself, in the absence of melatonin, produced no effect on growth of MCF-7 cells. This finding is consistent with our control data.

To test whether melatonin-mediated growth inhibition was blocked by 12-mG magnetic fields over a broad range of concentrations, we conducted studies at 10^{-11} – 10^{-5} M melatonin. Figure 5 reveals that a 12-mG magnetic field can significantly inhibit melatonin's action across the concentration range of 10^{-11} – 10^{-7} M ($p < 0.02$). This 12-mG effect on melatonin is reproducible in our laboratory; to date in 100% (seven out of seven) of our experiments showing significant melatonin inhibition at 2 mG, we have observed blocking by the 12-mG field.

Inhibition of Tamoxifen Action by a 12-mG, 60-Hz Magnetic Field

Extending our melatonin studies to tamoxifen, we tested the effects of 60-Hz, 2- and 12-mG fields on the growth-inhibitory action of tamoxifen over a range of doses, from 10^{-6} – 10^{-8} M. This range includes

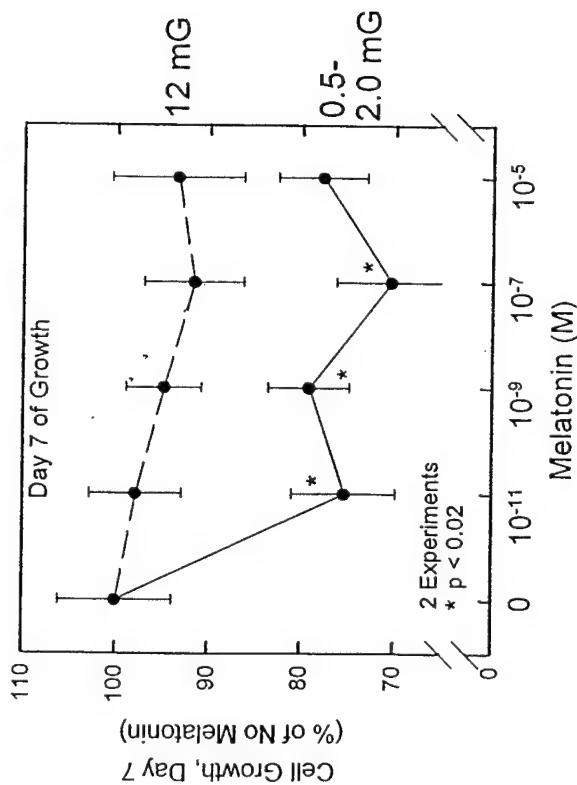


FIG. 5. Effect of a 12-mG versus a 0.5- to 2-mG (environmental background) magnetic field on inhibition of MCF-7 cell growth by melatonin, across a range of doses. Cell growth was determined by counting on day 7; all numbers are expressed as percentage of control (untreated) growth. Results are the means \pm SE of two experiments. The Student *t*-test shows a significant effect of the 12-mG field versus the 2-mG field at each individual melatonin dose for 10⁻¹¹–10⁻⁷ M melatonin ($p < 0.02$) (comparison of 0.5–2.0 mG versus 12 mG at individual melatonin doses); multifactor analysis of variance shows a significant 12-mG blocking effect ($p < 0.0001$) across the entire concentration range of hormone.

tamoxifen's pharmacological dose of 150 ng/mL, corresponding to 4×10^{-7} M (Swain and Lippman 1990).

Figure 6 shows cell growth data (day 7) for experiments involving a 2-mG versus a 12-mG magnetic field; cell growth is shown normalized to 100% for MCF-7 cells in the absence of tamoxifen. In a 2-mG field, tamoxifen inhibited MCF-7 cell growth in a dose-dependent manner; there was approximately 68% inhibition at 10⁻⁶ M tamoxifen, decreasing to 38% and 1% at 10⁻⁷ and 10⁻⁸ M, respectively. These data agree

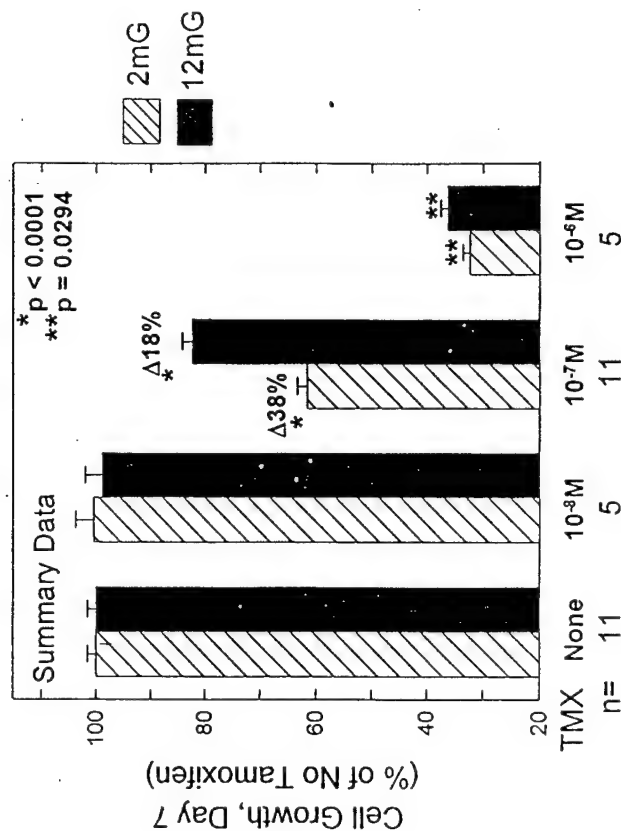


FIG. 6. Effect of a 12-mG versus a 2-mG magnetic field on inhibition of MCF-7 cell growth on day 7 by 10⁻⁸–10⁻⁶ M tamoxifen (TMX). In all experiments, cells were grown within mu-metal shields. Results are the means of 5 or 11 experiments.

with previous reports of tamoxifen's in vitro growth-inhibitory activity on MCF-7 cells (Lippman et al. 1976).

The growth inhibitory action of 10⁻⁷ tamoxifen was seen to be reduced or partially blocked by the presence of a 12-mG magnetic field. In a 12-mG field, only an 18% reduction of cell growth by day 7 was observed compared to a 38% reduction in the presence of a 2-mG field. However, the 12-mG field has only a slight effect on the action of 10⁻⁶ M tamoxifen (from 68% inhibition to 64%; $p = 0.0294$). This dose-dependent effect may be due to a threshold response, or to toxicity of the higher dose of tamoxifen. As with melatonin, the field interaction

requires the presence of tamoxifen; but it differs from that for melatonin (Fig. 5), which appears to be essentially constant across doses of 10^{-5} – 10^{-11} M.

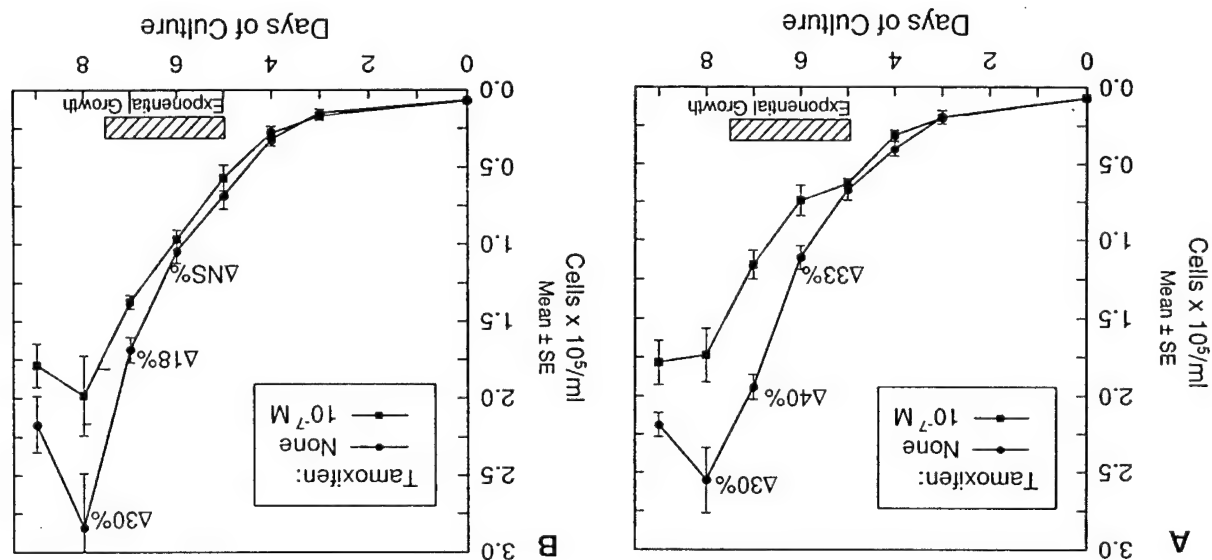
It could be argued that this result represents a temporary field effect that is observed perhaps only on day 7. However, this 12-mG blocking effect was seen on both days of tamoxifen sensitivity during MCF-7 exponential growth (compare Figs. 7A and 7B). Furthermore, these results are reproducible in our laboratory: In 10 of 11 experiments in which we have seen tamoxifen significantly inhibit MCF-7 cell growth, we have observed a significant ($p < 0.05$) blocking effect by the 12-mG magnetic field.

These data support an effect of a 12-mG, environmental-level magnetic field on tamoxifen's growth-inhibitory action in vitro at pharmacological doses. Recently, follow-on studies have been performed in our laboratory in which the experimenter was blinded to tamoxifen drug treatment; a similar 12-mG magnetic field effect was observed as shown in Figure 6 for tamoxifen at 10^{-7} M.

The Magnetic Field is Associated with the Field Blocking Effect

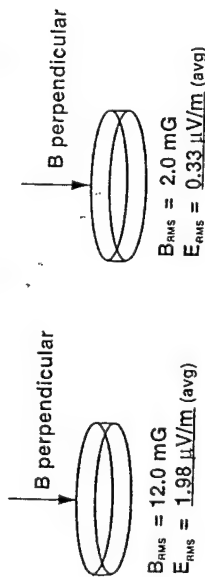
An important question to address, and one of significant mechanistic importance, is referred to in our laboratory as the "E versus B" question. This question relates to whether either the magnetic field (B) or the induced electric field (E) component is responsible for the blocking effect of both melatonin and tamoxifen. According to Faraday's Law of Current Induction, a time-varying magnetic field will induce in an object an electric field proportional to the radius of the cross-sectional area perpendicular to the incident magnetic field (Bassen et al. 1992; Liburdy 1992a, 1994b). Therefore, in order to differentiate E-field and B-field effects on cell growth, we simultaneously exposed MCF-7 cells in three matched incubators to either a 2-mG magnetic field, a 12-mG magnetic field, or a second 12-mG magnetic field rotated 90° (B-field vector parallel to culture dish). As shown in Figure 8, rotating the 12-mG field 90° reduces the effective induced E field nearly 5.6-fold from an induced E field [root mean square (RMS)—average] component of $1.98 \mu\text{V/m}$ to $0.353 \mu\text{V/m}$ by reducing the cross-section seen by the B field when rotated, while maintaining a constant 12-mG B field

FIG. 7. Growth curve of MCF-7 cells in the presence or absence of 10^{-7} M tamoxifen. Exponential growth occurs on days 5, 6, and 7. Panel A: Growth in a 2-mG magnetic field. Tamoxifen shows 33% and 40% inhibition on days 6 and 7, respectively. Panel B: Growth in a 12-mG magnetic field. Tamoxifen exhibits 0% and 18% inhibition on days 6 and 7, respectively.



E vs. B: Is the Induced Electric Field or the Applied Magnetic Field the Metric?

$$[E_{\text{RMS}} = \pi r B_{\text{RMS}}; r = \text{radius}/2 = r_{(\text{avg})}]$$



Rotate 12-mG Field to Reduce E_{RMS} (avg) by 5.6-fold.

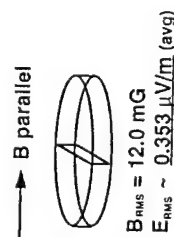


Fig. 8. Estimated values of average induced E (electric) fields in 2-mG (perpendicular), 12-mG (perpendicular), and 12-mG (parallel) magnetic field exposure systems, based on Faraday's Law of Current Induction. The magnetic B field exposure (B_{RMS}) at two different orientations remains the same for the MCF-7 monolayer culture; however, the average induced E field [E_{RMS} (avg)], which depends on the cross-sectional area of the culture media containing electrolytes seen by the B field, is reduced approximately 5.6-fold when the 12-mG field is rotated 90° from the B perpendicular to the B parallel orientation.

(M. Misakian, personal communication¹). The induced E field is expressed as the RMS value of the sinusoidally varying induced E field computed according to Faraday's Law of Current Induction using the average radius of the Petri dish, radius/2. The induced E field in the parallel orientation is essentially uniform over the surface of the dish upon which the cells are located [$E_{\text{RMS}} = \sim 0.353 \mu\text{V/m(avg)}$].¹ This is

different from the case in which the magnetic field is in the perpendicular orientation, where E varies according to the radius of the dish via Faraday's Law; the average electric field corresponds to radius/2, as indicated in Figure 8. To reduce E, we could instead have reduced dish radius, but this method would introduce potential biological complications due to alterations in surface/volume ratios and cell distribution. Such alterations have been shown in modeling studies to have a significant effect on the induced current density in cell monolayers exposed to a perpendicular 60-Hz magnetic field (M. Misakian, personal communication; see footnote on p. 686). Addressing the E versus B question is important from a mechanistic point of view: Magnetic fields can penetrate cells, but electric fields induced by environmental-level magnetic fields cannot penetrate beyond the cell membrane. Therefore, induced electric fields most likely interact directly with the cell at the membrane level (Liburdy 1992a, b).

The results are presented in Figures 9 and 10 for melatonin and tamoxifen, respectively. As we have reported previously (Liburdy et al. 1993b), magnetic fields did not affect MCF-7 cell growth significantly in the absence of melatonin. However, across three experiments, growth was significantly inhibited by 10^{-9} M melatonin, for an average of 52% inhibition on day 7 in a 2-mG magnetic field ($p < 0.0001$). In the 12-mG field oriented perpendicular to the plane of cells, melatonin's activity was blocked nearly completely ($p = 0.6493$); in the 12-mG magnetic field rotated 90° relative to the plane of the plate, melatonin's action was still blocked significantly ($p = 0.1101$). These data suggest that the 12-mG magnetic field component is the operative one for melatonin blocking effects.

In Figure 10, the analogous experiment for tamoxifen shows similar results. With 10^{-7} M tamoxifen in a 2-mG magnetic field, MCF-7 cell growth was significantly inhibited on day 7 by an average of 40% across four experiments ($p < 0.0001$). In a 12-mG magnetic field, the MCF-7 cell growth was still significantly blocked ($p = 0.0032$), but was reduced to an average of 15% inhibition. Similar results were seen in the 12-mG magnetic field rotated 90°, with an average of 17% inhibition (no significant difference from 12-mG perpendicular field; $p = 0.7506$). Thus, the 12-mG magnetic field component is associated with blocking tamoxifen as well as melatonin inhibition of MCF-7 cell growth.

¹ The calculated electric fields in the Petri dishes do not take into account the presence of cell proximity effects as described by Drs. M. Stuchly and W. Xi in, "Modeling induced currents in biological cells exposed to low frequency magnetic fields," *Phys. Med. Biol.* 39:1319-1330, 1994.

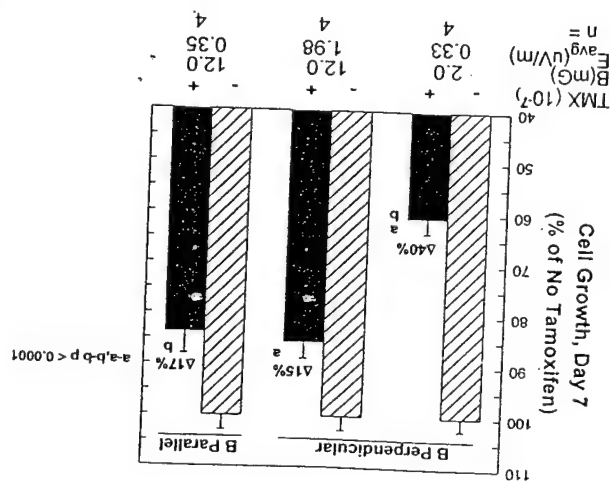


Fig. 10. Effect of 60-Hz magnetic field orientation on tamoxifen (TMX) cytotatic action in MCF-7 cells on day 7. The cells in the 2-mG field show an average of 40% inhibition; the 12-mG perpendicular and parallel cultures show 15% and 17% inhibition, respectively.

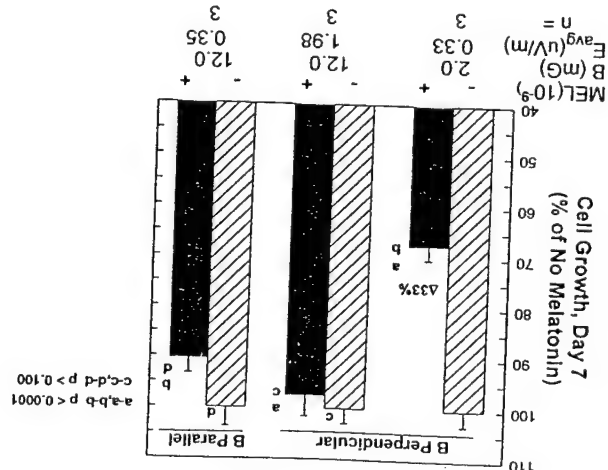


Fig. 9. Effect of the 60-Hz magnetic field orientation on melatonin's cytotatic action in MCF-7 cells. Cells were counted after 7 days in culture with or without melatonin (MEL) treatment in a 2-mG perpendicular field, a 12-mG perpendicular field, or a 12-mG parallel field. All values are expressed as a percent of the untreated culture cell counts in the same field. The cells in the 2-mG field show an average of 33% inhibition by 10⁻⁹ M melatonin; in the 12-mG perpendicular and 12-mG parallel fields, inhibition is reduced to 2% and 9%, respectively.

DISCUSSION

In studies in our laboratory, reviewed here, we have observed that environmental-level, 12-mG, 60-Hz magnetic fields partially block tamoxifen's cytotatic action, and completely block melatonin's cytotatic action on human breast cancer cells *in vitro*. We believe these findings may represent the first experimental report of environmental-level EMF interfering with the cytotatic action of a hormone or drug *in vitro*.

The tamoxifen data raise the possibility that the estrogen receptor may be a site for magnetic field interaction with the cell, since tamoxifen is an anti-estrogen that specifically binds to the estrogen receptor (Coezy et al. 1982; Martin et al. 1988). This hypothesis is speculation, and we caution that tamoxifen is reported to alter the metabolism of cells that are estrogen receptor-negative, which implies that this drug is multifactorial in action. In addition, we find differences in dose-response relationships between the magnetic field effect for tamoxifen versus melatonin, suggesting possible differences in an interaction mechanism between the two compounds. Finally, unlike many EMF effects reported in the literature, we find that the magnetic field itself, not the induced electric field, is associated with this effect (Liburdy 1995).

For both tamoxifen and melatonin, there are a number of possible levels at which a magnetic field interaction might take place: at the membrane, at the estrogen receptor, at various other signal-transduction molecules, at the nuclear membrane, or during transcription or translation necessary for regulation of cell growth and division. At each level, the magnetic field, which penetrates the cell, could act specifically or nonspecifically. For example, at the MCF-7 cell membrane, magnetic fields could alter either drug/hormone entry, or calcium (Ca²⁺) entry, influencing downstream signal-transduction events to overcome cytotatic effects. In support of this latter hypothesis, some magnetic field exposures have been reported to elevate intracellular Ca²⁺ levels in cells (Liburdy 1995, 1994b, 1992a-c; Liburdy et al. 1993c, d; Waliczek and Liburdy 1990), and intracellular Ca²⁺ concentration plays a role in estrogen receptor expression in MCF-7 (Ree et al. 1991). The regulation of MCF-7 cell growth is complex; "crosstalk" occurs between the two signal-transduction mechanisms of the estrogen

gen receptor and of calcium-dependent signaling regulated by growth factors at the cell surface (Philips et al. 1993).

Far more is known about the mechanism of tamoxifen activity in MCF-7 cells than that of melatonin. Melatonin may bind to a specific receptor, and several groups are investigating this possibility (Acuña-Castroviejo et al. 1994; Menendez-Pelaez and Reiter 1993; Ebisawa et al. 1994), but this putative binding site has not yet been isolated in MCF-7 cells. Melatonin has been reported to suppress the transcription of the estrogen receptor gene in MCF-7 cells (Molis et al. 1994); thus, there may be an indirect link between melatonin, the estrogen receptor, and melatonin's oncostatic properties. Tamoxifen, on the other hand, is an anti-estrogen that specifically binds to the estrogen receptor (Swain and Lippman 1990). It is thought that tamoxifen inhibits MCF-7 cell growth by inducing an alternate conformational change in the estrogen receptor (different from that caused by estradiol), which allows the receptor complex to bind to its DNA-binding regions but not transcribe its estrogen receptor response genes (Martin et al. 1988). Therefore, while the magnetic field could interfere with tamoxifen entry through the cell membrane, our data could also be explained by a field-associated modulation of tamoxifen binding to the estrogen receptor. However, the 12-mG field does not affect MCF-7 cell growth in the absence of tamoxifen, suggesting that the magnetic field may alter tamoxifen but not estradiol binding to the estrogen receptor, or may differentially affect binding of the estrogen receptor complex to its DNA-binding domain. Alternatively, the field could alter estrogen receptor gene expression in the cell, allowing the cell to overcome tamoxifen inhibition of cell growth at intermediate tamoxifen doses but not at higher ones. This possibility would explain the minimal field effect seen at 10^{-6} M tamoxifen. It would be interesting to test magnetic field effects on ICI 164,384 (a pure anti-estrogen), because tamoxifen has partial agonist activities (Chalbos et al. 1993; Gottardi et al. 1989); the field may favor tamoxifen's agonist activity.

In contrast, the mechanism of melatonin's cytostatic effect is as yet unknown, but melatonin may also interact with the estrogen receptor. Melatonin blocks estradiol's mitogenic effect in MCF-7, and estradiol partially rescues MCF-7 from melatonin inhibition (Cos et al. 1991; Danforth et al. 1983). The differential magnetic field effect on mela-

tonin versus tamoxifen (dose-independent versus dose-dependent) could be due to differences in the mechanism of interaction with the two compounds, in the mechanism of the two compounds themselves, or in toxicity. If tamoxifen is highly toxic at 10^{-6} M, in contrast to melatonin, magnetic field interactions could not rescue the killed cells.

Our observation that the magnetic field, not the electric field, is associated with blocking of both melatonin and tamoxifen has some importance regarding mechanisms. Induced E fields in the cell culture media interact initially at the level of the cell membrane, as they cannot penetrate beyond the cell membrane at power-line frequencies (Liburdy 1992a, b). The B field, however, while able to act at the cell membrane, penetrates the cell, providing more extensive possibilities for a site of interaction.

The question of E or B fields as the operative component is important from a second standpoint—that of environmental dosimetry in recommending safe exposure levels for humans. Most previous studies of biological effects of EMF have found the E field (Liburdy 1995), and not the B field to be the operative metric, and investigators have argued that an induced E field biological effect is unlikely at milligauss magnetic field strengths, since the resultant induced E field is less than the "thermal noise limit" within the cells (minimum response threshold of 10^{-4} V/m) (Weaver and Astumian 1990). However, we note that our *in vitro* model system is not a true representation of *in vivo* conditions.

One biophysical model that could explain how a magnetic field interacts with biological systems is the presence of a magnetic sensor within the cell (such as magnetite). Such a sensor could theoretically translate magnetic fields in the milligauss range into biomechanical effects (Kirschvink et al. 1992, 1993; Kirschvink 1992a; Polk 1994). Depending where such a sensor is physically located, effects on ion channels, receptors, or protein-protein interactions might be influenced. Currently, there is no direct experimental evidence for the presence of magnetite in MCF-7 cells.

A second biophysical model for magnetic field interaction with target molecules relates to magnetochimistry and radical pair recombination. Strong static magnetic fields (1000–1500 G) recently have been shown to alter a kinetic parameter, V_{\max}/K_m , of a biochemical reaction, most likely through modification of intersystem crossing rates

between singlet and triplet spin states in the spin-correlated radical pair (Harkins and Grissom 1994). Recently, in a collaborative effort between Dr. L. Packer's laboratory and ours, a report has suggested that specialized cells (such as neutrophils) which generate large quantities of free radicals upon activation ("respiratory burst") produce significantly more free radical species when activated in the presence of a 1-G, 60-Hz magnetic field (Roy et al. 1995). Although these studies are of interest, the fields involved are orders of magnitude higher than those employed in our studies. Regarding free radicals and melatonin, we note the interesting recent observations based on *in vivo* and *in vitro* experimental evidence that melatonin can act as a strong radical scavenger (Reiter et al. 1993).

Recently, a hypothesis was discussed that weak, low-frequency EMF may couple to biological systems via a mechanism involving stochastic resonance (Weissenfeld and Moss 1994). Although speculative, as the authors state, if stochastic resonance is relevant, the effects of a weak magnetic field might be amplified to have biological ramifications.

The studies reported here open the door for future studies to test possible mechanisms of field interaction, including studies to measure drug and hormone uptake and localization within the cell, ligand-receptor binding, binding to estrogen receptor response elements, estrogen receptor expression, cell-cycle kinetics, and signal-transduction parameters (such as G-proteins, intracellular Ca^{+2} , c-myc, and c-erb). A fruitful line of research would be to extend the findings reported here to other estrogen receptor-positive mammary lines in order to determine the generality of the effect. Estrogen receptor-negative cells could be evaluated as well as controls. Certain biophysical questions also need further attention, such as assessing a dose threshold for magnetic field strengths, frequency dependence, exposure time dependence, and reversibility. Such studies could lead to a better understanding of the mechanism of environmental magnetic field modulation of hormone/drug interactions at the cellular level.

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23 Epidemiologic Studies of EMF and Breast Cancer Risk: A Biologically Based Overview

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INTRODUCTION

Electric and magnetic fields (EMF) associated with the electric power we ordinarily use in houses, offices, and factories are ubiquitous. A

ENVIRONMENTAL MAGNETIC FIELDS INHIBIT THE ANTIPROLIFERATIVE ACTION OF TAMOXIFEN AND MELATONIN IN A HUMAN BREAST CANCER CELL LINE

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ABSTRACT: We have previously reported that environmental-level magnetic fields (1.2 μ Tesla (12mG), 60 Hz) block the growth inhibition of the hormone melatonin (10^{-9} M) on MCF-7 human breast cancer cells, in vitro. We now report that the same 1.2 μ Tesla, 60Hz magnetic fields significantly block the growth inhibitory action of pharmacological levels of tamoxifen (10^{-7} M). In biophysical studies we have taken advantage of Faraday's Law of Current Induction and tested whether the 12mG magnetic field (B) or the associated induced electric field (E) is responsible for this field effect on melatonin and tamoxifen. We observe that the magnetic field component is associated with the field blocking effect on melatonin and tamoxifen function. To our knowledge the tamoxifen studies represent the first experimental evidence for an environmental-level magnetic field modification of drug interaction with human breast cancer cells. Together, these findings provide support to the theory that environmental-level magnetic fields can act to modify the action of a drug or hormone on regulation of cell proliferation. Melatonin and tamoxifen may act through different biological pathways to down-regulate cell growth, and further studies are required to identify a specific biological site of interaction for the 1.2 μ Tesla magnetic field.

KEYWORDS: MCF-7, tamoxifen, melatonin, magnetic fields, cell proliferation, human breast cancer cells.

ABBREVIATIONS: Hz, hertz; mG, milligauss; B field, magnetic field; E field, electric field; AC, alternating current (time-varying magnetic field); DC, direct current (static magnetic field); rms, root mean square; ER, estrogen receptor; DMBA, dimethylbenz(a)anthracene; IR, infrared.

1. INTRODUCTION

One biological effect of low-frequency, time-varying electric and magnetic fields that has been reported by several investigators is the depression of secretion of the hormone melatonin from the pineal gland into the blood stream. This effect, first reported by B. Wilson in rats [Wilson et al., 1981; Wilson et al., 1983; Wilson et al., 1986], has since been observed in cultured pinealocytes [Welker et al., 1983], hamsters [Yellon, 1994], and has been reported in abstract form in some human volunteers exposed to 200mG, 60 Hz magnetic fields at night [Graham et al., 1993, 1994]. These observations, in conjunction with the finding that melatonin can provide protection against breast cancer in animal models [Subramanian and Kothari, 1991] has led to a hypothesis proposed by Dr. Richard Stevens that magnetic fields may increase risk of breast cancer [Stevens et al., 1992]. Melatonin is known to have a spectrum of biological functions including immune function enhancement and oncostatic properties [Yu and Reiter, 1993; Brzezinski, 1997]. Of particular interest is melatonin's inhibition of DMBA-induced rat mammary gland carcinogenesis [Subramanian and Kothari, 1991]. Consistent with an *in vivo* model of magnetic field interaction involving suppression of melatonin secretion from the pineal gland, recent animal studies by Drs. W. Loscher and M. Mevissen have reported that magnetic fields can enhance DMBA-induced breast cancer cell growth in rats in a dose-dependent manner [Loscher et al., 1993; Mevissen et al. 1993]. In *in vitro* studies Dr. D. Blask has demonstrated that melatonin at physiological levels inhibits MCF-7 human breast cancer cell growth [Hill and Blask, 1988; Cos and Blask, 1990; Cos et al., 1991], further supporting the oncostatic properties of melatonin. Using MCF-7 cells obtained from Dr. Blask we have confirmed Blask's original observation that melatonin inhibits MCF-7 cell growth, and we have reported experimental evidence for a magnetic field interaction with MCF-7 cells: continuous exposure to environmental-level 1.2 μ Tesla, 60 Hz magnetic fields block melatonin's growth inhibitory action on MCF-7 cells, while having no significant effect on untreated cells [Liburdy et al., 1993c; Liburdy et al., 1993d]. Three laboratories have independently reported results in abstract form that are consistent with this magnetic field effect on melatonin [Blask et al., 1993a; Blask et al., 1993b; Blackman et al., 1996; Luben et al., 1996].

To investigate a possible biological mechanism for such a magnetic field effect, we have employed the antiestrogen tamoxifen to ask whether such fields decrease its growth inhibitory action. Tamoxifen, the most widely used antiestrogen therapy for the control of breast cancer, induces an alternate conformational change in the estrogen receptor (ER) upon binding [Martin et al., 1988], which allows the receptor complex to bind to its DNA-binding regions but not transcribe its ER response genes. In biophysical studies, we have addressed the question of whether the magnetic field itself, or the induced electric field associated with the magnetic field exposure, is critical for the field effect involving melatonin or tamoxifen. To carry out these studies and test for a magnetic or electric field dependence, we have employed a cell culture exposure system utilizing a mu-metal shielding chamber that generates a uniform magnetic field [Liburdy, 1994; Liburdy, 1995] and we have oriented (rotated by 90°) the magnetic field vector so that the induced electric field is significantly reduced according to Faraday's Law of Current Induction.

2. MATERIALS AND METHODS

Cell Culture Techniques.

MCF-7 cells [Soule et al., 1973] at passage 18 were a generous gift of Dr. David Blask of the Mary Imogene Bassett Hospital Research Institute, Cooperstown, NY. Cells were maintained in a monolayer and passed as described [Liburdy et al., 1993c]; fetal bovine serum (product #101, lot #10786) was obtained from Tissue Culture Biologicals, Tulare, CA. For tamoxifen sensitivity assays, MCF-7 cells (passages 25-37) were harvested in 0.2% EDTA phosphate buffer (2g/liter Na₂-EDTA, 8 g/liter NaCl, 0.2 g/liter KH₂PO₆, 1.15 g/liter Na₂HPO₆), dispersed by passing three times through a 25 gauge needle, and seeded at 0.1×10^5 cells/35mm dish in 1.5ml of media. After cell attachment, media was changed, with or without chemical treatment. Tamoxifen (Sigma Product #T9262) and melatonin (n-acetyl-5-methoxytryptamine; Sigma Product #M5250) solutions were prepared in minimum ethanol, followed by serial dilution in media (final ethanol concentrations are approximately 0.001% and 0.00001% for tamoxifen and melatonin, respectively). On counting days, triplicate plates were harvested with trypsin solution at 37°C (0.50g/liter trypsin, 0.5g/liter EDTA, 1.0g/liter glucose, and 0.58 g/liter NaHCO₃) and counted by hemacytometer.

Magnetic Field Exposure System

Cells were exposed continuously during growth curves using the cell culture exposure system shown in Figure 1 [Liburdy, 1994; Liburdy, 1995]. We employed several such exposure systems so that simultaneous experiments could be conducted on the same cells but at different field strengths. Special features are: 1) a perforated Plexiglas platform table, 2) a four-coil Merritt exposure system (plastic frame wound with double-wrap, bifilar cable, turn ratio of 26/11/11/26) [Merritt et al., 1983; Kirschvink, 1992a], 3) a ventilated mu-metal chamber (Co-Nectic AA shielding (1mm), Magnetic Shield Corporation, Perfection Mica Co., Bensenville, IL) to eliminate extraneous magnetic fields, 4) a water-jacketed incubator (Queue Systems, Inc., Parkersburg, WV, Model 2710), maintained at $37 \pm 0.1^\circ\text{C}$, and 5) the ability to rotate the Merritt coil 90° so that the magnetic field vector is rotated from a standard perpendicular orientation to a horizontal orientation which significantly reduces the induced electric field without altering the magnetic field flux density experienced by the cells. Field dosimetry was performed as described [Liburdy et al., 1993c; Liburdy, 1995]. Our protocol requires that field readings are taken before and after experiments; values were within approximately $\pm 5\%$. Static (DC) fields were reduced to approximately $0.01 \mu\text{Tesla}$ by the mu-metal chambers. Temperature inside the mu-metal chambers was monitored with thermistor probes (YSI Inc., Yellow Springs, OH) placed adjacent to cell culture plates. Measurement of CO_2 levels inside of our mu-metal chambers where cells are cultured have been performed using a) a remote IR sensing probe, and b) a remote thermocouple sensing probe, and both indicate that CO_2 levels inside the chambers are maintained at 5% CO_2 (Incubator Services, Barnesville, OH).

Statistical analyses

Data were tested for statistical significance using the SigmaPlot Student t-test (Jandel Corporation, Corte Madera, CA). All error bars in the figures represent the standard error of the mean.

3. RESULTS

Inhibition of Tamoxifen Action by a 12mG, 60Hz Magnetic Field

Figure 2 presents experimental data showing the effect of 60Hz, $0.2 \mu\text{Tesla}$ or $1.2 \mu\text{Tesla}$ magnetic fields on tamoxifen's growth inhibition of MCF-7 cells over a range of doses (from 10^{-6}M to 10^{-8}M). This range includes tamoxifen's pharmacological dose of 150 ng/ml , corresponding to $6 \times 10^{-7}\text{M}$ [Swain and Lippman, 1990]. Cell growth on day seven is shown

normalized to 100% for untreated MCF-7 cells. At 0.2 μ Tesla, tamoxifen inhibits cell growth in a dose-dependent manner: exhibiting 68% inhibition at 10^{-6} M tamoxifen, decreasing to 40% and 1% at 10^{-7} M and 10^{-8} M, respectively. These data agree well with previous reports of tamoxifen's in vitro growth inhibitory activity on MCF-7 cells [Lippman et al., 1976]. In a 1.2 μ Tesla magnetic field, the growth inhibitory action of 10^{-7} M tamoxifen is reduced significantly, from 40% to 17% ($p < 0.0001$). One of these twelve experiments involved blinding the experimenter to chemical treatment of cells with similar results obtained. Regarding the reproducibility of this effect in our hands, in eleven out of the twelve experiments in Figure 2 in which we have seen tamoxifen (10^{-7} M) inhibit MCF-7 cell growth, we have observed a significant ($p < 0.05$) blocking effect by the 1.2 μ Tesla magnetic field. Interestingly, the 1.2 μ Tesla field was observed to have no significant effect on 10^{-6} M tamoxifen (68% vs. 66%). There may exist a tamoxifen dose-threshold response that depends on the level of toxicity displayed by tamoxifen on MCF-7 cells at higher doses.

Since it could be argued that the results presented in Figure 2 might represent a field effect that is observed only on day seven of cell growth, we conducted experiments in which cell growth was followed for nine days. This data is shown in Figures 3a (0.2 μ Tesla data) and 3b (1.2 μ Tesla data). The 1.2 μ Tesla blocking effect was seen on both days of tamoxifen sensitivity during MCF-7 exponential growth (compare Figures 3a and 3b).

The Magnetic Field is Associated with the Field Blocking Effect

We next asked whether the magnetic field itself or the induced electric field component is responsible for this blocking effect of tamoxifen, as reported here, as well as the blocking effect we have previously reported for melatonin [Liburdy et al., 1993c; Liburdy, et al., 1993d]. According to Faraday's Law of Induction, a time varying magnetic field will induce an electric field in an object proportional to the radius of the cross-sectional area perpendicular to the incident magnetic field [Bassen et al., 1992; Liburdy, 1992a]. Therefore, to differentiate E field and B field effects on cell growth, we simultaneously exposed MCF-7 cells in three matched incubators to either a 0.2 μ Tesla magnetic field, a 1.2 μ Tesla magnetic field, or a second 1.2 μ Tesla magnetic field, rotated 90° (with the field direction parallel to the plate surface). This exposure situation along with magnetic field and induced electric field exposure values are depicted in Figure 4. Rotating the 1.2 μ Tesla field 90° reduces the effective cross-section seen

by the magnetic field and diminishes the induced E field nearly 5.6-fold (from an average induced E field component of $1.96\mu\text{V/m}$ to $0.353\mu\text{V/m}$, while maintaining a constant $1.2\mu\text{Tesla}$ B field [M. Misakian, personal communication: Stuchly and Xi, 1994]. The electric field induced by the parallel magnetic field is essentially uniform over the entire dish surface ($E_{\text{rms}} = \sim 0.353\mu\text{V/m}$). In the perpendicular magnetic field, however, the induced electric field varies with the radius of the dish via Faraday's law; the average electric field corresponds to $\text{radius}/2$.

In Figure 5 are shown results for MCF-7 cell growth in the presence of tamoxifen (10^{-7}M) and a $0.2\mu\text{Tesla}$ magnetic field; MCF-7 cell growth was significantly inhibited on Day 7 by an average of 40% across four experiments ($p < 0.0001$). In a $1.2\mu\text{Tesla}$ magnetic field, MCF-7 cell growth was also significantly blocked ($p = 0.0032$), but was reduced to 15% inhibition. Similar results were seen in the $1.2\mu\text{Tesla}$ magnetic field rotated 90° , with an average of 17% inhibition (no significant difference from $1.2\mu\text{Tesla}$ perpendicular field; $p = 0.7506$). Thus, the $1.2\mu\text{Tesla}$ magnetic field component is associated with blocking tamoxifen inhibition of MCF-7 cell growth.

In analogous studies we tested whether the blocking effect of a $1.2\mu\text{Tesla}$ magnetic field on melatonin action [Liburdy et al., 1993c; Liburdy et al., 1993d], was associated with the magnetic field or the induced electric field. In Figure 6 are presented the results of these studies. In the absence of melatonin, as we have reported previously, magnetic fields did not affect MCF-7 cell growth significantly. However, across three experiments, growth was significantly inhibited by 10^{-9}M melatonin, for an average of 33% inhibition on Day 7 in a $0.2\mu\text{Tesla}$ magnetic field ($p < 0.0001$). When the $1.2\mu\text{Tesla}$ field was oriented in the standard position perpendicular to the plane of cells, melatonin's activity was blocked nearly completely ($p = 0.6493$). When the $1.2\mu\text{Tesla}$ magnetic field was rotated 90° relative to the plane of the plate, melatonin's action was still blocked significantly ($p = 0.1101$). This data suggests that the $1.2\mu\text{Tesla}$ magnetic field component is associated with blocking melatonin's cytostatic action.

4. DISCUSSION

In this study, we observe that environmental-level $1.2\mu\text{Tesla}$, 60Hz magnetic fields partially block tamoxifen's growth inhibitory action on human mammary tumor (MCF-7) cells in vitro.

This finding extends our original observation that a 1.2 μ Tesla, 60Hz magnetic field blocks melatonin's growth inhibitory action on MCF-7 cells. Unlike many electromagnetic field effects reported in the literature [Liburdy, 1995], we find the magnetic field itself, not the induced electric field, is associated with these field effects.

Induced E fields in the cell culture media interact initially at the cell membrane, as they cannot penetrate beyond the cell membrane at power-line frequencies. The B field, however, penetrates the cell, increasing the possibilities for a biological site of interaction: signal-transduction molecules (including the estrogen receptor), the nuclear membrane, transcription or translation events necessary for cell growth and division. One simple interpretation of our data is that magnetic fields might inhibit tamoxifen or melatonin entry into the cell; although unlikely, this is a testable hypothesis. Alternatively, a magnetic field might influence one or more of tamoxifen's actions. Tamoxifen is a multiphasic drug and as an antiestrogen it binds to the ER, but it also has other biological effects such as interacting with calmodulin and protein kinase C to inhibit their functions [Taylor et al., 1984]. Such interactions might be influenced by a magnetic field leading to a blockage of tamoxifen's growth inhibitory action. Tamoxifen is also reported to have partial agonist activities [Fujimoto and Katzenellenbogen, 1994], such as a stimulation of uterine tissue growth in animals which might be influenced by magnetic fields. It is also possible that the magnetic field acts non-specifically relative to tamoxifen: calcium entry might be enhanced to trigger downstream signal transduction events which overcome tamoxifen's growth inhibitory effects. Calcium is a potentially interesting indicator for future studies since a) some magnetic field exposures have been reported to elevate intracellular Ca^{+2} levels [Walleczek and Liburdy, 1990; Liburdy, 1992a; Liburdy, 1992b; Liburdy, 1992c; Liburdy et al., 1993a; Liburdy et al., 1993b; Liburdy, 1995] and b) intracellular Ca^{+2} concentration is believed to play a role in ER expression in MCF-7 [Ree et al., 1991].

In contrast to tamoxifen, the hormone melatonin has been shown to influence human physiological functions including the biological regulation of circadian cycles and sleep, and there is evidence that melatonin may also influence reproduction, tumor growth, and aging [Yu and Reiter, 1993; Brzezinski, 1997]. Studies investigating a mechanism of action have identified two membrane-bound melatonin binding sites: ML1 (high affinity, picomolar) and ML2 (low affinity, nanomolar) [Morgan et al., 1994; Dubocovich, 1995]. ML1 receptors belong to the

family of guanosine triphosphate-binding proteins (G protein-coupled receptors) [Acuna-Costroviejo et al., 1994; Ebisawa et al., 1994], and activation of these receptors results in the inhibition of adenylate cyclase activity in target cells. It is believed that these receptors are involved in retinal function, circadian rhythms, and reproduction. ML2 melatonin receptors are coupled to the stimulation of membrane phosphoinositide hydrolysis and signal transduction and may play a role in regulating cell growth; their tissue distribution has not been determined. Melatonin is also reported to bind to target molecules inside the cell. Melatonin can bind calmodulin and may directly affect calcium signaling [Benitez-King and Anton-Fay, 1993]. Melatonin is also reported to bind a family of nuclear retinoid Z receptors, suggesting that melatonin may affect nuclear events during hormone signaling [Becker-Andre et al., 1994]. As of yet it is not known whether melatonin receptors, and which type, are present in MCF-7 cells.

Investigators have suggested that an induced E field associated with μ Tesla magnetic field strengths may not lead to biological effects since the resultant induced E field is less than the "thermal noise limit" within the cells (minimum response threshold of 10^{-6} V/m) [Weaver and Astumian, 1990]. We have shown in this study that inhibition of melatonin's and tamoxifen's action is associated with the magnetic field itself. Several different biophysical models have been hypothesized to describe how a magnetic field might interact with biological systems: the presence of a magnetic sensor(s) within the cell (such as magnetite) [Kirschvink et al., 1992; Kirschvink et al., 1993; Polk, 1994]; free radical magnetochemistry [Reiter et al., 1993; Harkins and Grissom, 1994; Scaiano et al., 1994; Frankel and Liburdy, 1995; Roy et al., 1995]; stochastic resonance [Wiesenfeld and Moss, 1994]; and biological electron transfer [see abstract, Nair and Liburdy, 1996]. At present a consensus among researchers has not been achieved regarding a specific biophysical interaction to explain "environmental-level" magnetic field bioeffects. However, independent evaluation of such bioeffects [Blask et al., 1993a; Blask et al., 1993b; Blackman et al., 1996; Luben et al., 1996] represents one important step in building a solid biological database and in identifying a model system with which biophysical models can be tested.

Recently, experiments were conducted in our laboratory in collaboration with Dr. Stefan Engstrom to test whether the magnetic field inhibition of tamoxifen function, described here, is associated with a relatively fast or a relatively slow interaction timescale [see abstract, Harland et

al., 1996]. This question is important in assessing whether the transduction step is an isolated biophysical process or if it is an integral part of a more complex biological structure involving relatively long natural timescales. The findings from these collaborative studies provide support for a relatively slow interaction timescale on the order of milliseconds. This timescale is consistent with a physical transductive step strongly coupled to a biological process, e.g. receptor binding and translocation. Such a timescale is also consistent with certain interaction mechanisms (e.g., parametric resonance [Blackman et al., 1995; Prato et al., 1995; Engstrom, 1996]) but does not support others (e.g. free radical recombination mechanisms).

In the future, studies are needed to identify a possible biologically based interaction site(s), and to assess critical field parameters (e.g. frequency, field intensity threshold, exposure duration dependence). One potentially promising approach to identify receptor involvement is the use of pure antiestrogens, e.g. ICI 182,780, that bind specifically to the estrogen receptor, to test for field effects on ER binding. Use of other specific biochemical agents that bind to calmodulin and PKC to block function may also prove useful. In addition, the use of secondary cell lines derived from MCF-7 parent cells and other human mammary tumor cell lines may provide important information about the biological site of interaction; for example, there are human mammary epithelial cell types which do not express the estrogen receptor but are tamoxifen sensitive.

Acknowledgments

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Figure Legends

Figure 1: Cell culture exposure system [23, 24]. Shown is the combination of the 4-square Merritt coil and the mu-metal chamber, which are both placed inside of a commercial cell culture incubator. Also shown is a thermistor temperature probe which is threaded through one ventilation hole into the mu-metal chamber and placed inside the chamber at the position where cell culture plates are typically located. The 4-square Merritt coil can be rotated 90° to reorient the magnetic field vector from the standard position in the vertical plane to orientation in the horizontal plane.

Figure 2: Effect of a 12mG versus a 2mG magnetic field on inhibition of MCF-7 cell growth on day 7 by 10^{-8} M to 10^{-6} M tamoxifen (TMX). In all experiments, cells were grown within mu-metal shields. Results are the means of five or twelve experiments.

Figure 3: Growth curve of MCF-7 cells in the presence or absence of 10^{-7} M tamoxifen. Exponential growth occurs on days 5, 6, and 7. **Panel A)** Growth in a 2mG magnetic field. Tamoxifen yields 33% and 40% inhibition, respectively, on days 6 and 7. **Panel B)** Growth in a 12mG magnetic field. Tamoxifen exhibits 0% and 18% inhibition on days 6 and 7.

Figure 4: Estimated values of average induced E (electric) fields in 2mG (perpendicular), 12mG (perpendicular), and 12mG (parallel) magnetic field exposure systems, based on Faraday's Law of Induction. The magnetic B field exposure (B_{rms}) at two different orientations remains the same for the MCF-7 monolayer culture; however, the average induced E field (E_{avg}), which depends on the crosssectional area of the culture media containing electrolytes seen by the B field, is reduced approximately 5.6-fold when the 12mG field is rotated 90° from the B perpendicular to the B parallel orientation.

Figure 5: Effect of 60Hz magnetic field orientation on tamoxifen (TMX) cytostatic action in MCF-7 cells on day 7. The cells in the 2mG field show an average of 40% inhibition; the 12mG perpendicular and parallel cultures show 15% and 17% inhibition, respectively.

Figure 6: Effect of the 60Hz magnetic field orientation on melatonin's cytostatic action in MCF-7 cells. Cells were counted after 7 days in culture with or without melatonin (MEL) treatment, in a 2mG perpendicular field, a 12mG perpendicular field, or a 12mG parallel field. All values are expressed as a percent of the untreated culture cell counts in the same field. The cells in the 2mG field show an average of 33% inhibition by 10^{-9} M melatonin; in the 12mG perpendicular and 12mG parallel fields, inhibition is reduced to 2% and 9%, respectively.

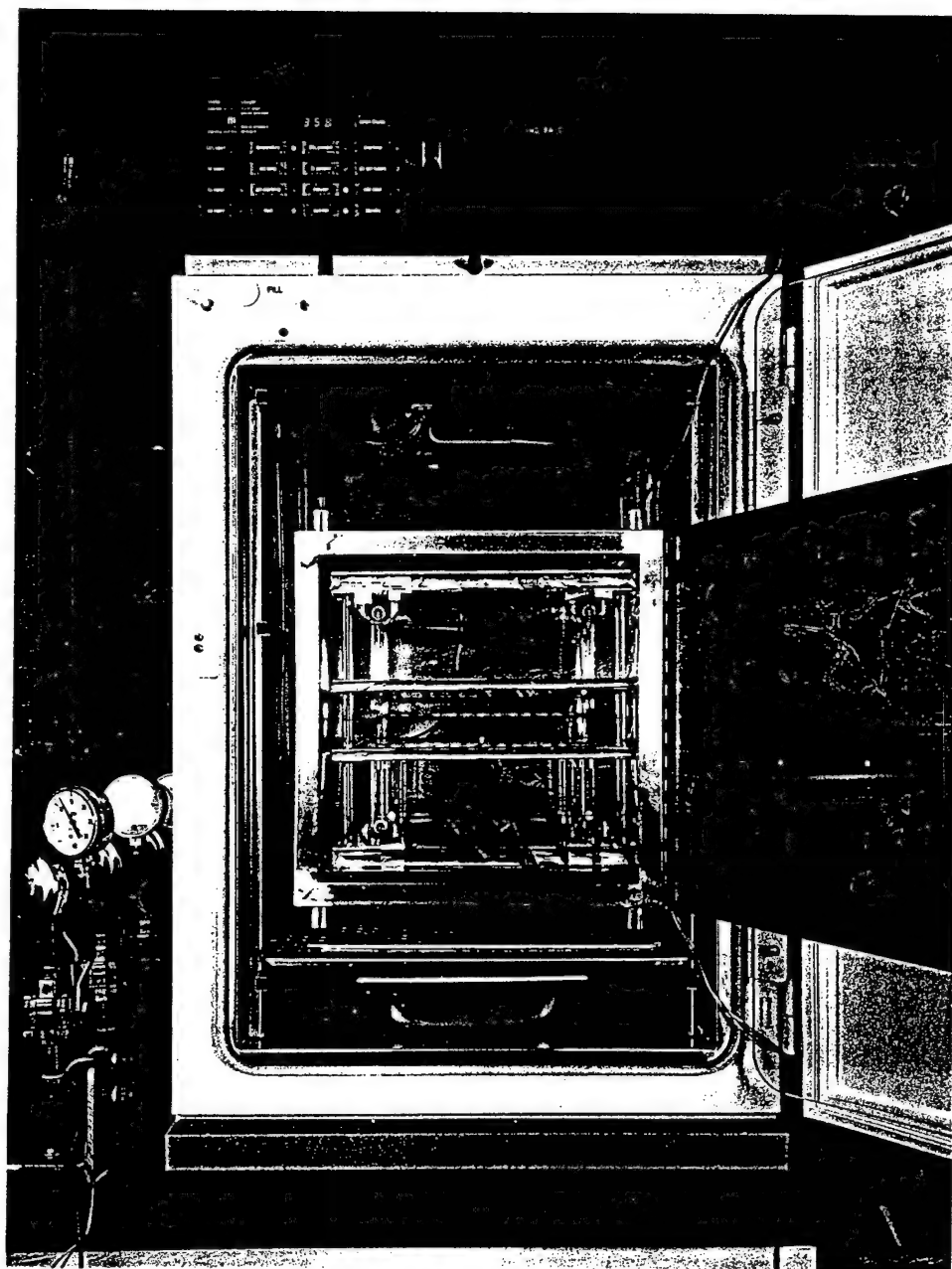


Figure 1

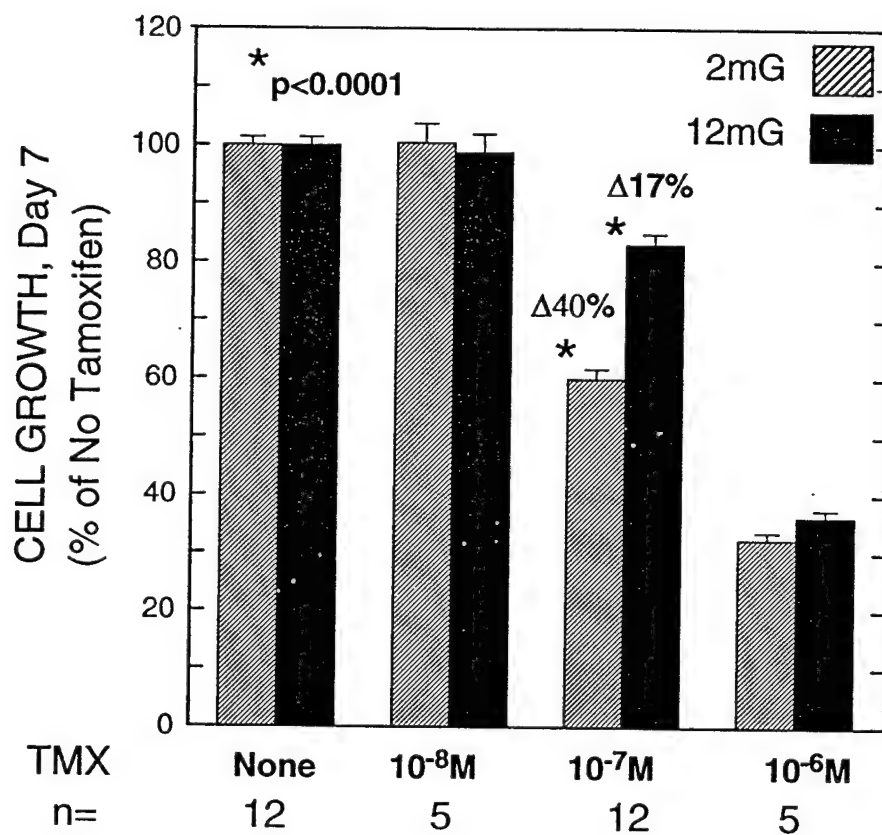


Figure 2

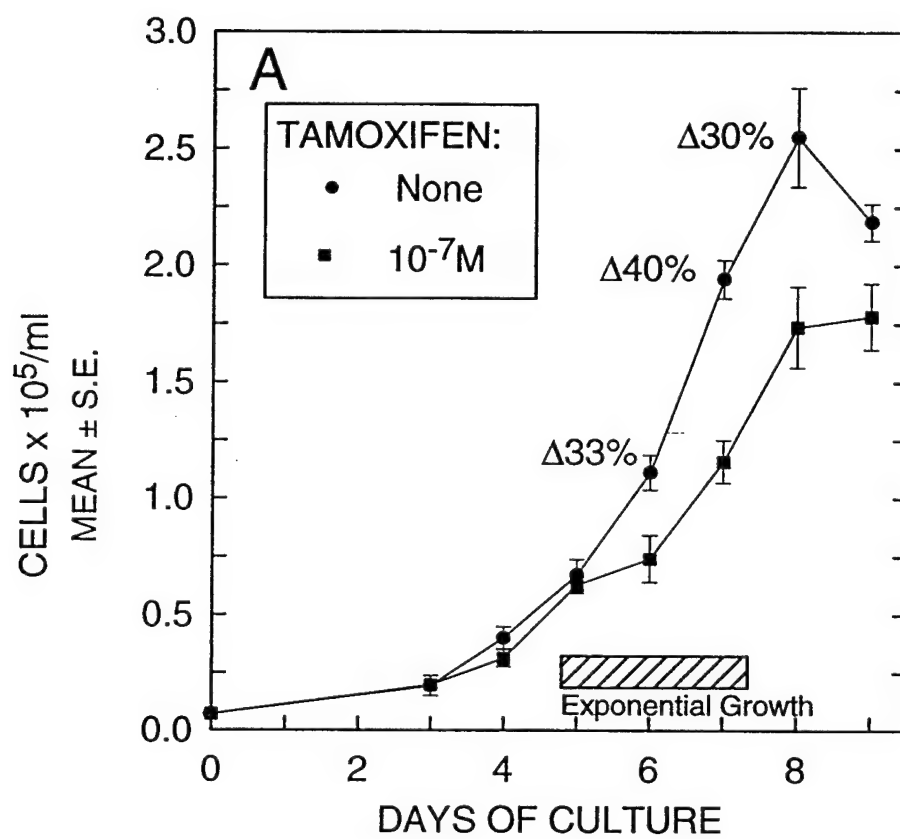


Figure 3A

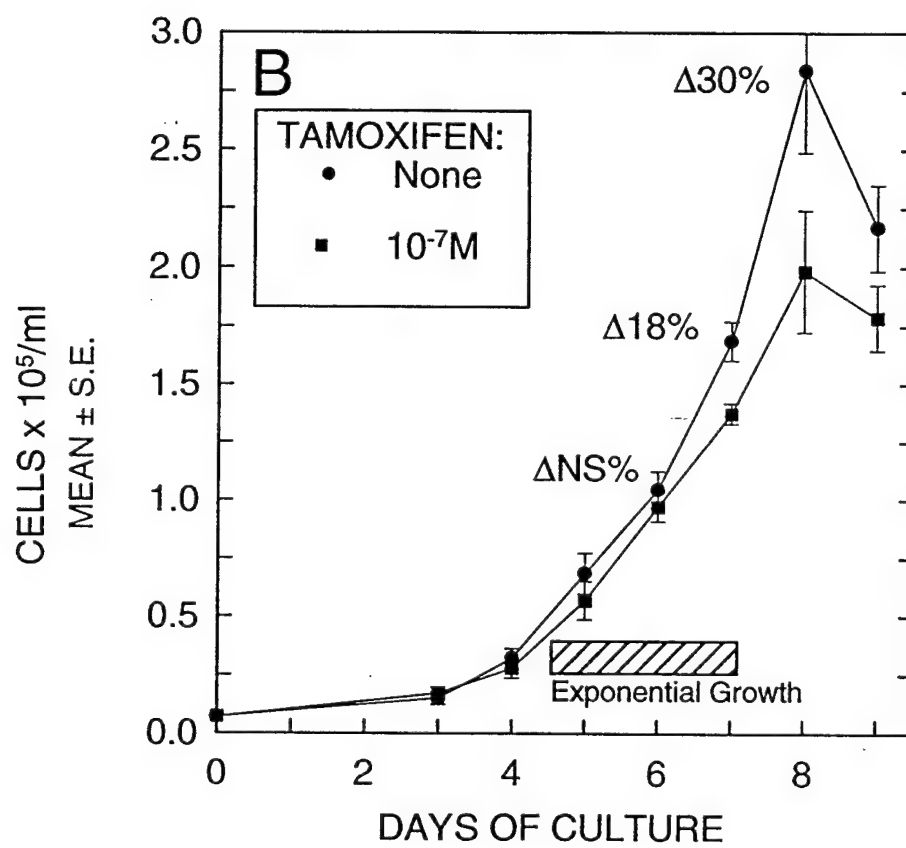
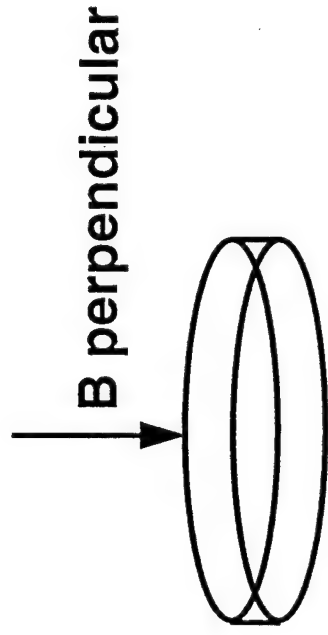


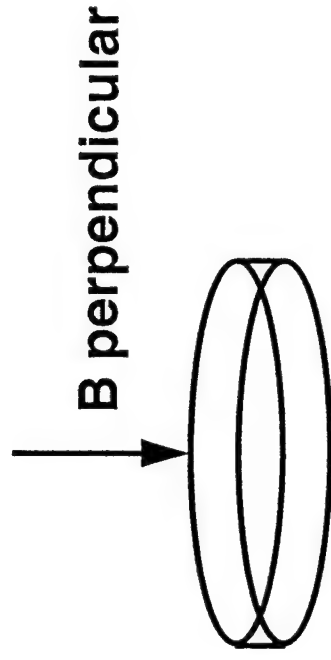
Figure 3B

$$E_{rms} = \pi f r B_{rms}; \quad r \text{ is radius}/2 = r(\text{avg})$$



$$B_{rms} = 12.0 \text{ mG}$$

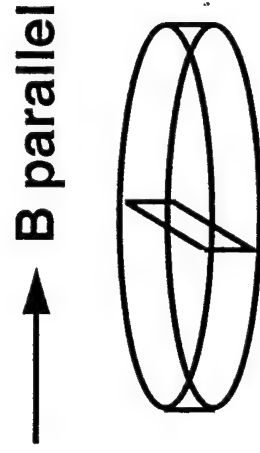
$$E_{rms} = \underline{1.96 \text{ uV/m(avg)}}$$



$$B_{rms} = 2.0 \text{ mG}$$

$$E_{rms} = \underline{0.33 \text{ uV/m(avg)}}$$

**Rotate 12mG Field
to Reduce $E_{rms}(\text{avg})$
by 5.6-fold.**



$$B_{rms} = 12.0 \text{ mG}$$

$$E_{rms} \sim \underline{0.353 \text{ uV/m(avg)}}$$

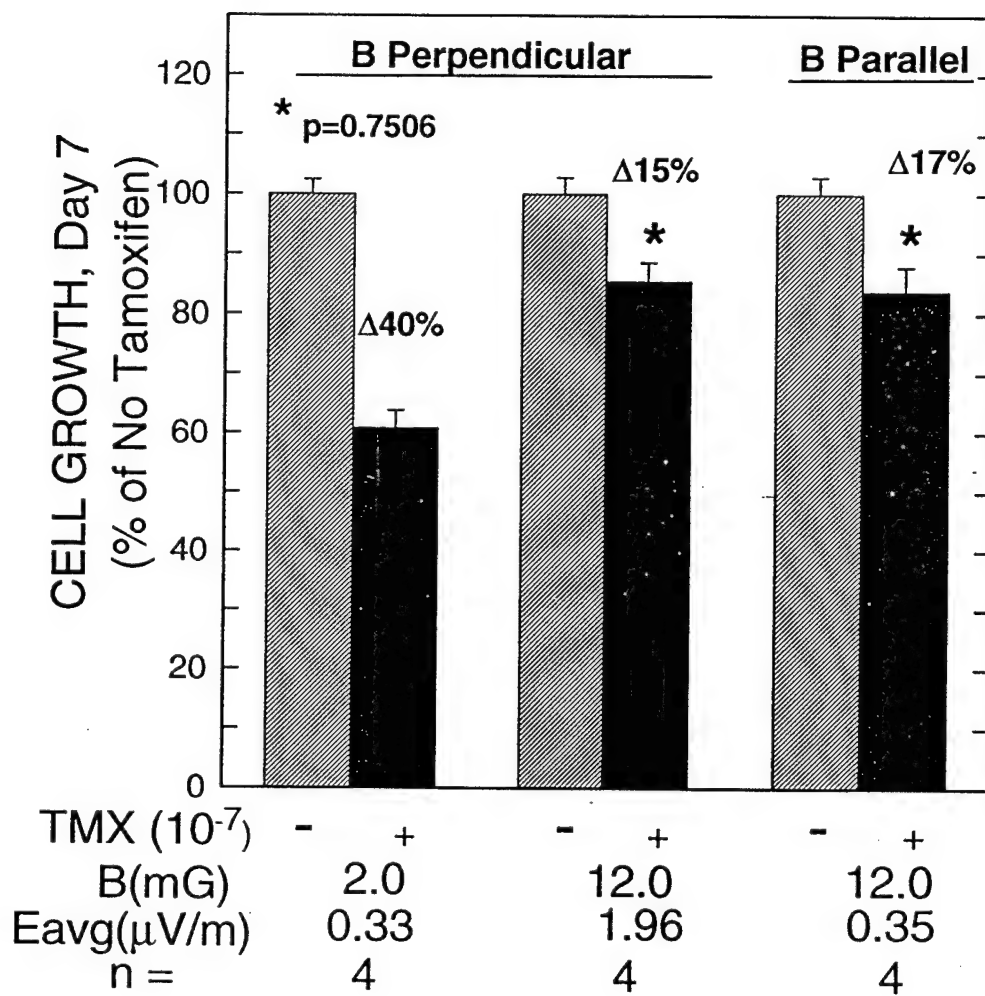


Figure 5

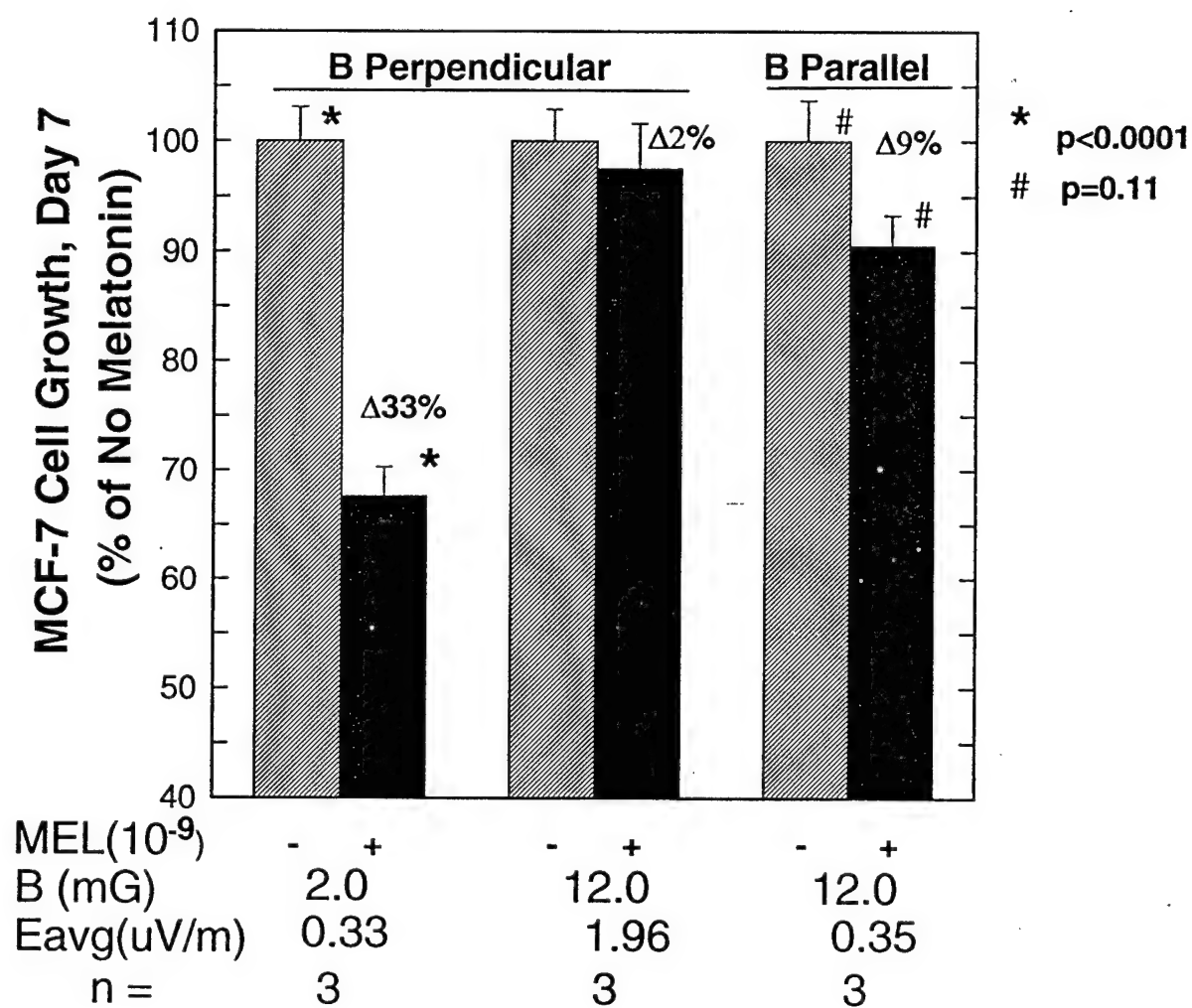


Figure 6

REPLICATION DATA

Blackman et al. (1996)

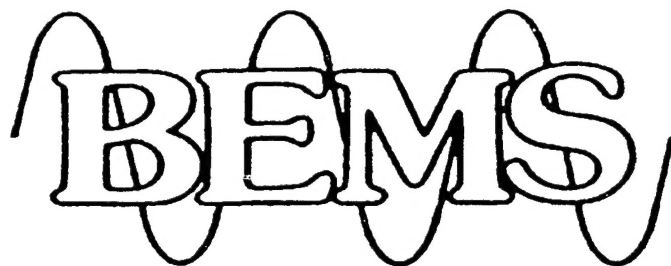
Independent replication of the 12mG effect on melatonin and MCF-7 cells in vitro.

Abst. A-1-2. BEMS Mtg., Victoria, CN

Luben et al. (1996)

Replication of 12mG EMF effect on melatonin responses of MCF-7 breast cancer cells in vitro.

Abst. A-1. DOE Mtg., San Antonio, TX



Abstract Book

Eighteenth Annual Meeting
Conference Centre, Victoria,
B.C., Canada

June 9-14, 1996



Courtesy of Tourism Victoria

TECHNICAL PROGRAM EIGHTEENTH ANNUAL MEETING

SESSION A-1: MELATONIN
CO-CHAIRS: Bary Wilson and Robert Liburdy

A-1-2

INDEPENDENT REPLICATION OF THE 12-MG MAGNETIC FIELD EFFECT ON MELATONIN AND MCF-7 CELLS IN VITRO. C.F. Blackman¹, S.G. Benane^{2*}, D.E. House^{2*} and J.P. Blanchard². ¹National Health & Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711, USA. ²Bechtel Corporation Research & Development, San Francisco, California 94119, USA.

Recent studies showed that physiological concentrations of melatonin (10^{-9} M) reduce the growth rate of transformed human breast cancer (MCF-7) cells in culture (1,2), but 12-mG; 60-Hz magnetic fields eliminate this melatonin-induced growth rate inhibition, while 2-mG, 60 Hz magnetic fields have no such effect (2,3).

OBJECTIVE: With the cooperation of the originating laboratory, our goal was to independently replicate these melatonin and magnetic field effects on MCF-7 cells (2).

APPROACH AND METHODS: We carefully followed the detailed protocol provided by the original laboratory which included using the same cells and lot of serum as in the original report. Mu-metal chambers screened ambient magnetic fields during treatments, allowing careful control of all fields to which the cells were exposed during testing. There were two deviations from the original protocol: a) our growth of stock MCF-7 cells was in a magnetically unshielded incubator, although the ambient 60-Hz fields were ≤ 2 mG as required by the protocol, and b) we used Helmholtz rather than Merritt coils to generate the magnetic fields during our tests. Three replications of each test were performed and each experiment employed a newly thawed vial of MCF-7 cells. Three dishes of cells were plated for each of three treatment conditions created in mu-metal boxes housed in 5% CO₂ incubators, as described below. Each treatment was continuous for 7 days. Cells were then harvested and counted in a blinded manner.

RESULTS: Combined results of the three experiments are:

	Control	Melatonin(MEL)	Melatonin(MEL) & MF
B Field	(<2 mG)	(<2 mG)	(12 mG)
mean	1.38	1.15	1.39
SE	0.15	0.14	0.14
n	9	9	9

where the mean is times 10^6 cells per ml. These results were analyzed by the REGWF multiple comparison procedure, the results of which indicated that the control and MEL & MF treatment means were not significantly different, but both means were significantly larger ($p < .001$) than the MEL mean.

DISCUSSION: These results independently confirm that a) melatonin can inhibit the growth of MCF-7 cells in culture (1), and, b) a 12-mG, 60-Hz magnetic field can completely block this oncostatic action (2). These results are particularly significant because: a) we believe our findings represent the first replication of a key magnetic field-induced bioeffect, and b) this foundation allows theorists to generate "testable" hypotheses to shed light on interaction mechanisms, both physical and biological in nature, using MCF-7 cell-based experimental data. The constructive communication established between our lab and the original lab lead to our ability to independently replicate their findings, a result which plays a critical role in scientific progress.

(1) Hill & Blask (1988) *Cancer Res.* 48:6121-6126.

(2) Liburdy, et al. (1993) *J. Pineal Research* 14: 89-97.

(3) Liburdy, et al. (1994) BEMS Mtg, Copenhagen, Abst. F-1-7.

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PROJECT ABSTRACTS

THE ANNUAL REVIEW OF RESEARCH ON BIOLOGICAL EFFECTS
OF ELECTRIC AND MAGNETIC FIELDS FROM
THE GENERATION, DELIVERY & USE OF ELECTRICITY

SAN ANTONIO, TEXAS, USA

NOVEMBER 19-21, 1996

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ABSTRACTS

CARCINOGENESIS: *IN VITRO* & *IN VIVO*

A-1

REPLICATION OF 12 mG EMF EFFECTS ON MELATONIN RESPONSES OF MCF-7 BREAST CANCER CELLS *IN VITRO*. R.A. Luben, S. Saraiya and A.P. Morgan. Division of Biomedical Sciences, University of California, Riverside, California 92521, USA.

INTRODUCTION: Epidemiological data have been interpreted as suggesting correlations between occupational exposure to elevated levels of power-frequency EMF and the incidence of breast cancer in humans. One major difficulty with this interpretation of the findings (and likewise with other findings possibly correlating EMF exposures with cancer incidence) is that no mechanism has been found which can account for carcinogenesis in cells exposed to EMF at the low field strengths encountered in environmental situations. Although there have been numerous reports of signal transduction-related responses in cells exposed to EMF *in vitro*, these have largely been confirmed to occur only at field strengths hundreds or thousands of times higher than the field strengths encountered in environmental exposures. However, Liburdy's group* has described an experimental system in which MCF-7 breast cancer cells appear to respond to environmental levels of EMF (circa 12 mG) in a manner consistent with increased cancer cell growth and/or decreased response to inhibitors of cancer cell growth. Independent replication of these findings has been reported by Blackman's group**.

OBJECTIVE: The objective of these studies was to replicate, with the cooperation of the originating laboratories, the studies conducted by Liburdy and Blackman.

METHODS: We obtained melatonin-sensitive MCF-7 cells, medium, serum, and protocols from Liburdy's lab and communicated with them on a regular basis. The same protocols used in Liburdy's lab were carefully followed for cell growth, cell counting, and data analysis. All experiments were carried out using double-blinding procedures throughout. Three identical incubators (60 Hz ambient magnetic fields 1.8-2.6 mG) were used for all cell propagation, experimental, and sham exposures. There were two differences between our experiments and those of Liburdy: 1) Mu-metal shields were not used in any of the incubators in our study; 2) Because the ambient magnetic field was already near 2 mG, the sham-exposed cells in our study were not subjected to an applied external magnetic field, but were grown under ambient magnetic field conditions in an activated, zero-field Merritt coil. [Note: Blackman** also used ambient fields for his controls.] All treatments were carried out continuously for the duration of the experiment.

RESULTS: Four experiments were done; one of these was rejected because cell growth ceased (probably due to contamination) after day 3. The results obtained in the remaining three experiments were consistent and statistically significant in each case. Growth was measured by cell counts at days 5 and 6.

1) In sham exposed cells (1.8-2.6 mG ambient field), 10^{-9} M melatonin caused a mean 10.2% DECREASE ($p < 0.03$) in cell growth as compared to non-melatonin-treated controls. This is consistent with previous results reported by Liburdy*, Blackman** and others.

2) Exposure of cells to 12 mG 60 Hz magnetic field produced no significant change in the growth of non-melatonin-treated cells ($p > 0.3$); but cells treated with 10^{-9} melatonin plus 12 mG EMF showed an 18% INCREASE ($p < 0.003$) in growth.

3) The net 28% difference between (melatonin + EMF) and (melatonin - EMF) cell growth could not be accounted for by differences in cell viability, cell density, or incubator location.

DISCUSSION: We found that exposure of breast cancer cells to 12 mG 60 Hz EMF induced a reproducible net increase (mean +28%, $p < 0.001$) in the growth rate of MCF-7 cells treated with a physiological dose of melatonin. This constitutes a replication of the observations reported previously by Liburdy* and Blackman**, in that EMF produced a blocking of the anti-cell-growth effects of melatonin. There are some variations between our findings and theirs: we saw a significant increase in growth rate of (melatonin + EMF) treated cells, which was not reported previously; and the amount of inhibition by melatonin of non-EMF-treated cell growth was smaller in our experiments than previously reported (although still statistically significant). It is possible that these differences were due to clonal differences in the strains of MCF-7 cells used, or possibly to the precise combination of ambient fields, applied fields, and shielding (or lack thereof), which has varied slightly among the replications. Nevertheless, the net differences between (melatonin + EMF) and (melatonin - EMF) groups is both qualitatively and quantitatively consistent in all the studies. This